Improving the diagnosis of tuberculosis in hard to diagnose populations: Clinical evaluation of GeneXpert MTB/RIF and alternative approaches in Ethiopia

Dissertation for the degree of doctor in Biomedical Sciences at the University of Antwerp to be defended by

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Faculty of Pharmaceutical,
Biomedical and Veterinary Sciences
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Verbetering van de diagnose van tuberculose bij moeilijk te diagnosticeren populaties: Klinische evaluatie van GeneXpert MTB/RIF en alternatieve benaderingen in Ethiopië

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Abbreviations/ Acronyms

AFB	Acid-fast bacilli
BTB	Bovine tuberculosis
BSC	Biological safety cabinet
CDR	Case Detection Rate
CNR	Case Notification rate
CSF	Cerebrospinal fluid
CI	Confidence interval
Ct	Cycle threshold
CRS	Composite reference standard
DOTS	Directly Observed Treatment Short course
DST	Drug susceptibility testing
EPTB	Extrapulmonary tuberculosis
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FIND	Foundation for Innovative and New Diagnostics
FNA	Fine needle aspiration
FNAC	Fine-needle aspiration cytology
FMOH	Federal Ministry of Health
HEWs	Health Extension Workers
HIV	Human Immunodeficiency Virus
HFs	Health facilities
IFN-y	Interferon gamma
IGRA	Interferon gamma release assay
FM	Fluorescent Microscopy
ITM	Institute of Tropical Medicine
IRB	Institutional review board
JUSH	Jimma University Specialized Hospital
JU-MRC	Jimma University-Mycobacteriology Research Center
L-J	Löwenstein-Jensen
LED	Light emitting diode
LTBI	Latent tuberculosis infection
MDR-TB	Multidrug- resistant TB

MGIT	Mycobacteria Growth Indicator Tube
MTB	Mycobacterium tuberculosis
MTBc	Mycobacterium tuberculosis complex
MIRU-VNTR	Mycobacterial interspersed repetitive units of variable- number tandem repeats
NAATs	Nucleic acid amplification tests
LF-LAM	Lateral flow-urine lipoarabinomannan assay
LAM	Lipoarabinomannan
LPA	Line probe assays
NALC	N-acetyl-L-cysteine
NTM	Non-tuberculous mycobacteria
NPV	Negative predictive value
NTLCP	National Tuberculosis and Leprosy Control Programme
NTCP	National TB Control Program
OADC	Oleic acid-albumin-dextrose-catalase
PNB	Para-nitrobenzoic acid
PTB	Pulmonary TB
PANTA	Polymyxin B, amphoterisin B, nalidixic acid, trimethoprim, azlocillin
PCR	Polymerase chain reaction
POC	point-of-care
PPV	Positive predictive value
RIF	Rifampicin
RR-TB	rifampicin-resistant TB
SNP	Single nucleotide polymorphism
SOPs	Standard Operating Procedures
TB	Tuberculosis
TBLN	Tuberculous lymphadenitis
TSR	Treatment Success Rate
TST	Tuberculin skin test
WHO	World Health Organization
XDR-TB	Extensively drug resistant TB
ZN	Ziehl-Neelson

Summary

In resource-limited settings like Ethiopia, conventional smear microscopy is the most commonly used diagnostic tool for detection of tuberculosis (TB). In 2014, the case detection rate in Ethiopia was about 60%, leaving high rates of undiagnosed TB, leading to patient's not receiving TB treatment and ongoing spread of TB in the community. Patients with sputum smear-negative, extrapulmonary (EP) or drug-resistant TB are particularly affected by the failure of microscopy as primary diagnostic tool. Improving TB detection rates and further reducing the burden of disease in Ethiopia will require evaluation and optimization of new diagnostic technologies as well as introduction of alternative approaches for improving the performance of current laboratory techniques. In this PhD thesis, we investigated such tools and alternative strategies for smear-negative pulmonary (P) and EPTB in Ethiopia.

We evaluated the performance of Xpert MTB/RIF for the diagnosis of smear-negative PTB patients, and approaches to improve its sensitivity by performing a second Xpert MTB/RIF for each patient or pre-treating sputum with bleach followed by centrifugation (Chapter three). Over the study period, 185 presumed smear-negative PTB patients were eligible and provided two sputum specimens (spot and morning). Our results indicate that Xpert MTB/RIF has modest sensitivity (63%) and excellent specificity (98%) for smear-negative culture-positive cases and substantially increases the yield of confirmed smear-negative PTB cases. However, incremental yield from the second Xpert MTB/RIF per patient was insignificant, while treating smear-negative sputum with bleach followed by centrifugation and testing the pellet significantly improved Xpert MTB/RIF sensitivity from 63% to 74% in our study, though its added value needs further study on a larger scale.

Diagnosis of EPTB poses a major challenge. A more accurate test to diagnose various forms of EPTB that can easily be incorporated in the routine TB control program, would contribute significantly towards improving EPTB case-detection and thus reducing the morbidity and mortality. In the second study (Chapter four) we documented that concentration of fine needle aspirate (FNA) significantly improved the sensitivity of conventional smear-microscopy for the diagnosis of TB lymphadenitis (TBLN) (35% on direct smear and 66% on concentrated smear), yet remains suboptimal. Cytology has excellent sensitivity (90%) but lowest specificity (45%). In areas where culture and Xpert MTB/RIF are not available, cytological diagnosis can be used in

conjunction with the smear-microscopy on concentrated smear for better laboratory confirmation of TBLN.

The diagnostic performance of fluorescent light-emitting diode (LED) microscopy for TBLN was studied on routinely collected FNAs from 144 presumptive TBLN cases (Chapter five). We found that the sensitivity of LED-microscopy (46%) was better than conventional microscopy (25%) but lower than cytology (82%). The specificity of LED-microscopy (90%) was comparable to that of conventional microscopy (94%) but higher than cytology (54%). LED-fluorescence microscopy requires a shorter examination time and will especially benefit high-workload clinics in high-burden TB countries like Ethiopia. Supplementing cytology with LED-microscopy can increase the specificity and improve management of presumed TBLN patients. Although LED-microscopy shows an improvement over conventional ZN, microscopy remains a suboptimal diagnostic technique, and search for better diagnostic tools remains imperative.

Hence, we evaluated the diagnostic accuracy of Xpert MTB/RIF in concentrated FNA samples for the diagnosis of TBLN (Chapter six). A total of 143 consecutive presumptive TBLN patients were enrolled in this study. Overall, the results indicate that Xpert MTB/RIF can diagnose TBLN better than conventional methods (FNA cytology and microscopy), with excellent sensitivity (88%) and specificity (91%) compared to Löwenstein-Jensen culture. Moreover, Xpert MTB/RIF offers rapid detection of rifampicin-resistant TBLN directly from the clinical sample, an important advantage over cytology, smear microscopy and culture. In agreement with World Health Organization (WHO) endorsement, we conclude that Xpert MTB/RIF is an easy and suitable method to be used in TB endemic settings and may be the optimal first line test for the diagnosis of TBLN in routine clinical practice.

In Ethiopia, Xpert MTB/RIF is undergoing phased implementation for routine diagnosis of TB and drug resistance. However, there are no comprehensive data on the diagnostic utility of Xpert MTB/RIF for presumed EPTB cases to guide clinical practice in Ethiopia. Having promising results of Xpert MTB/RIF on concentrated FNA samples, we further investigated the diagnostic accuracy of Xpert MTB/RIF for the detection of EPTB by site of disease (Chapter seven). A total of 572 extra-pulmonary specimens were collected from patients with clinically presumed EPTB. These comprised 279 lymph node specimens, 45 cerebrospinal fluids (CSFs), and 248 other fluid specimens (159 pleural, 80 peritoneal and 9 pericardial). The diagnostic accuracy of Xpert MTB/RIF was calculated compared to a composite reference standard (CRS). We found

excellent specificity of Xpert MTB/RIF across different sample types (pooled specificity of 98%), highlighting its utility as a rule-in test for EPTB diagnosis. In contrast, the sensitivity was extremely heterogeneous among the specimen types (30% - 90%), with highest sensitivity seen when testing lymph nodes, modest sensitivity for CSF and lowest sensitivity for other specimens such as pleural and peritoneal fluids.

We conclude that Xpert MTB/RIF may be used as initial diagnostic test for testing lymph node specimens from patients suspected of having TBLN. While Xpert MTB/RIF has modest sensitivity in CSF, it could significantly improve the diagnosis of TB meningitis as it provides TB diagnosis within two hours. A negative Xpert MTB/RIF test on fluid specimens (pleural and peritoneal) does not exclude the diagnosis of EPTB and patients with a high clinical probability should still be started on anti-TB treatment, despite a negative Xpert MTB/RIF result. Moreover, Xpert MTB/RIF detected rifampicin- resistance in 13 patients in perfect agreement with line probe assay and hence improves management of drug resistant EPTB patients.

In the last study of this doctoral thesis, we explored the genetic diversity of circulating *M. tuberculosis* complex (MTBc) strains isolated from 304 TBLN patients in Southwest Ethiopia (Chapter eight). We found that the TBLN in this region was caused by a wide diversity of MTBc strains with predominance of the Ethiopian specific sub-lineages within Lineage 4 followed by Lineage 3 and the Haarlem sub-lineage 4. Our analysis also revealed the presence of the typical Ethiopian Lineage 7 in Southwest Ethiopia. Despite some minor difference in frequency, the overall MTBc strain distribution in Southwest Ethiopia is similar to the observed distribution among pulmonary and TBLN patients in other regions of Ethiopia, indicating successful transmission of TB across the country by human movement. Moreover, the contribution of *M. bovis* in TBLN infection is minimal, confirming earlier findings that in Ethiopia TBLN arise from the same source as pulmonary TB, rather than from an external zoonotic source.

The results of this doctoral thesis improve our understanding of different diagnostic methods and approaches for effective diagnosis of TB in hard to diagnose groups such as EPTB and smear-negative PTB. Xpert MTB/RIF has better diagnostic performance than the currently available conventional diagnostic tools and its integration into a routine diagnostic algorithm must be considered in Ethiopia. Based on our findings, we recommend that Xpert MTB/RIF

should be used as a replacement test for usual practice including conventional microscopy and cytology for testing lymph node specimens from patients suspected of having TBLN in Ethiopia.

The maximum benefit from Xpert MTB/RIF testing can be obtained by targeted testing of patients such as presumed to have TB lymphadenitis or TB meningitis. Future research should focus on evaluating potential impact beyond diagnostic accuracy such as patient outcomes, cost effectiveness, scalability and effects of programmatic implementations. It became evident that no form of smear-negative or EPTB can rely on a single diagnostic test. Any chosen diagnostic method or approach should be based on a thorough assessment of the local TB case finding system and gaps. A major global effort is needed to develop simple, fast and more sensitive point of care TB diagnostic test that can be readily implemented in resource-limited settings.

Sumenvatting

In lage-inkomst landen zoals Ethiopië, is direct microscopisch onderzoek de meest gebruikte diagnostische methode voor het opsporen van tuberculose (tbc). In 2014 werd op die manier ongeveer 60% van de tbc gevallen gedetecteerd, waardoor grote aantallen niet-gediagnosticeerde tbc achterbleven. Deze kregen mogelijks geen correcte behandeling, met een verdere verspreiding van tbc in de gemeenschap als gevolg. Patiënten met een microscopie-negatief sputum resultaat, extra-pulmonale (EP) of geneesmiddelresistente tbc worden in het bijzonder getroffen door het falen van microscopie als primaire diagnostiek. Om de tbc-detectiegraad te verbeteren en de ziektelast in Ethiopië verder te verminderen, moeten nieuwe diagnosetechnieken worden geëvalueerd en geoptimaliseerd, evenals de introductie van alternatieve benaderingen om de prestaties van de huidige laboratoriumtechnieken te verbeteren. In dit proefschrift hebben we dergelijke analysemethodes en alternatieve strategieën voor microscopie-negatieve pulmonale (P) en EPTB in Ethiopië onderzocht.

We evalueerden de performante van Xpert MTB/RIF voor de diagnose van microscopie-negatieve PTB-patiënten en benaderingen om de gevoeligheid ervan te verbeteren door een tweede Xpert MTB/RIF uit te voeren voor elke patiënt of sputum voor te behandelen met bleekmiddel gevolgd door centrifugatie (Hoofdstuk drie). Gedurende de onderzoeksperiode verstrekten 185 veronderstelde microscopie-negatieve PTB-patiënten die in aanmerking kwamen twee sputum monsters (bij de consultatie en een ochtend sputum). Onze resultaten geven aan dat Xpert MTB/RIF een matige gevoeligheid (63%) en uitstekende specificiteit (98%) heeft voor microscopie-negatieve kweek-positieve gevallen, en het totaal van bevestigde microscopie-negatieve PTB-gevallen aanzienlijk verhoogt. De toegevoegde opbrengst van de tweede Xpert MTB / RIF per patiënt was echter niet significant, terwijl het behandelen van microscopie-negatief sputum met bleekmiddel gevolgd door centrifugatie en testen van de pellet de Xpert MTB / RIF-gevoeligheid aanzienlijk verbeterde van 63% tot 74% in ons onderzoek. Deze toegevoegde waarde moet verder worden bestudeerd op grotere schaal.

De diagnose van EPTB vormt een grote uitdaging. Een meer accurate test om verschillende vormen van EPTB te diagnosticeren die gemakkelijk kan worden geïntegreerd in het routinematige tbc-controleprogramma, zou aanzienlijk bijdragen tot het verbeteren van de detectiegraad voor EPTB en aldus de morbiditeit en mortaliteit verminderen. In het tweede onderzoek (Hoofdstuk vier) hebben we aangetoond dat de concentratie van fijne naald

aspiraten (FNA) de gevoeligheid van conventionele uitstrijkmicroscopie voor de diagnose van TB lymfadenitis (TBLN) significant verbeterde (35% op direct uitstrijkje en 66% op geconcentreerd uitstrijkje). Desondanks blijft de gevoeligheid suboptimaal. FNA-cytologie heeft een uitstekende gevoeligheid (90%) maar lage specificiteit (45%). In gebieden waar cultuur en Xpert MTB/RIF niet beschikbaar zijn, kan cytologische diagnose worden gebruikt in combinatie met microscopie op geconcentreerd uitstrijkjes voor een betere laboratoriumbevestiging van TBLN.

De diagnostische prestatie van fluorescente "light-emitting diode" (LED) microscopie voor TBLN werd bestudeerd op routinematig verzamelde FNA's uit 144 vermoedelijke TBLN-gevallen (Hoofdstuk vijf). We vonden dat de gevoeligheid van LED-microscopie (46%) beter was dan conventionele microscopie (25%) maar lager dan FNA-cytologie (82%). LED-fluorescentiemicroscopie vereist een kortere onderzoekstijd en zal vooral ten goede komen aan klinieken met een hoge werklast in zwaar belaste tbc-landen zoals Ethiopië. Het aanvullen van cytologie met LED-microscopie kan de specificiteit verhogen en het beheer van veronderstelde TBLN-patiënten verbeteren. Hoewel LED-microscopie een verbetering ten opzichte van conventionele ZN laat zien, blijft microscopie een suboptimale diagnostische techniek en blijft het zoeken naar betere diagnostische hulpmiddelen noodzakelijk.

Daarom evalueerden we de diagnostische accuraatheid van Xpert MTB/RIF in geconcentreerde FNA-monsters voor de diagnose van TBLN (Hoofdstuk zes). Een totaal van 143 opeenvolgende vermoedelijke TBLN-patiënten namen deel aan deze studie. Over het algemeen geven de resultaten aan dat Xpert MTB/RIF de TBLN beter kan diagnosticeren dan conventionele methoden (FNA-cytologie en microscopie), met een uitstekende gevoeligheid (88%) en specificiteit (91%) in vergelijking met de Löwenstein-Jensen cultuur. Bovendien biedt Xpert MTB/RIF een snelle detectie van rifampicine-resistente TBLN direct in het klinische monster, een belangrijk voordeel ten opzichte van cytologie, microscopie en cultuur. In overeenstemming met de goedkeuring door de Wereld Gezondheidsorganisatie concluderen we dat Xpert MTB/RIF een gemakkelijke en geschikte methode is om te worden gebruikt in tbc-endemische settings en mogelijk de optimale eerstelijns test is voor de diagnose van TBLN in de dagelijkse klinische praktijk.

In Ethiopië ondergaat Xpert MTB/RIF een gefaseerde implementatie voor routinematige diagnose van tbc en resistentie tegen geneesmiddelen. Er zijn echter geen uitgebreide

gegevens over het diagnostische nut van Xpert MTB/RIF voor veronderstelde EPTB-gevallen om de klinische praktijk in Ethiopië te begeleiden. Met veelbelovende resultaten van Xpert MTB/RIF op geconcentreerde FNA-monsters, onderzochten we de diagnostische nauwkeurigheid van Xpert MTB/RIF voor de detectie van verschillende klinische vormen van EPTB (Hoofdstuk zeven). Een totaal van 572 extra-pulmonale monsters werden verzameld van patiënten met klinisch veronderstelde EPTB. Deze omvatten 279 lymfklier monsters, 45 cerebrospinale vloeistoffen (CSF's) en 248 andere lichaamsvloeistoffen (159 pleura, 80 peritoneale en 9 pericardiale). De diagnostische nauwkeurigheid van Xpert MTB/RIF werd berekend in vergelijking met een samengestelde referentiestandaard (CRS). We vonden een uitstekende specificiteit van Xpert MTB/RIF voor verschillende types van monsters (gepoolde specificiteit van 98%), wat het nut als een 'rule-in' test voor de EPTB-diagnose aantoonde. Daarentegen was de gevoeligheid extreem heterogeen naargelang het soort monster (30% - 90%), met de hoogst waargenomen gevoeligheid bij het testen van lymfeklieren, een matige gevoeligheid voor CSF en de laagste gevoeligheid voor andere monsters zoals pleurale en peritoneale vloeistoffen.

We concluderen dat Xpert MTB/RIF kan worden gebruikt als initiële diagnostische test voor het testen van lymfklier monsters van patiënten met een vermoede TBLN. Hoewel Xpert MTB/RIF een bescheiden gevoeligheid heeft in CSF, kan het de diagnose van tuberculose-meningitis aanzienlijk verbeteren, aangezien het resultaat binnen twee uur gekend kan zijn. Een negatieve Xpert MTB/RIF-test op vloeistofmonsters (pleuraal en peritoneaal) sluit de diagnose van EPTB niet uit en patiënten met een hoge klinische waarschijnlijkheid moeten nog steeds worden gestart op anti-tbc behandeling, ondanks een negatief Xpert MTB/RIF-resultaat. Bovendien detecteerde Xpert MTB/RIF rifampicine resistentie bij 13 patiënten, in perfecte overeenstemming met de line-probe-assay en met een verbeterde opvolging van geneesmiddelresistente EPTB-patiënten tot gevolg.

In de laatste studie van dit proefschrift hebben we de genetische diversiteit onderzocht van circulerende *M. tuberculosis*-complex (MTBc) -stammen geïsoleerd uit 304 TBLN-patiënten in Zuidwest-Ethiopië (Hoofdstuk acht). We vonden dat de TBLN in deze regio werd veroorzaakt door een brede waaier van verschillende MTBc-stammen met overheersing van de Ethiopië-specifieke genetische lijnen binnen Lineage 4 gevolgd door Lineage 3 en de Lineage 4 Haarlem sub-lijn. Onze analyse onthulde ook de aanwezigheid van de typische Ethiopische Lineage 7 in

Zuidwest Ethiopië. Ondanks een klein verschil in frequentie, is de algehele verdeling van genetische MTBc types in Zuidwest Ethiopië vergelijkbaar met de waargenomen verdeling bij long- en TBLN-patiënten in andere regio's van Ethiopië, wat een indicatie is voor succesvolle overdracht van tbc door het hele land via menselijke beweging. Bovendien is de bijdrage van *M. bovis* aan de TBLN-infectie minimaal, wat eerdere bevindingen bevestigt dat in Ethiopië TBLN voortkomt uit dezelfde bron als pulmonale tuberculose eerder dan vanuit een externe zoönotische bron.

De resultaten van dit proefschrift verbeteren ons begrip van verschillende diagnostische methoden en benaderingen voor een effectieve diagnose van tbc in moeilijk te diagnosticeren groepen zoals EPTB en microscopie-negatieve PTB. Xpert MTB/RIF heeft betere diagnostische prestaties dan de momenteel beschikbare conventionele diagnostische hulpmiddelen, en de integratie ervan in een routine diagnostisch algoritme in Ethiopië moet worden overwogen. Op basis van onze bevindingen raden we aan Xpert MTB/RIF te gebruiken als vervangende test voor de tot nu toe gebruikelijke analyses waaronder conventionele microscopie en cytologie voor het testen van lymfkliermonsters van patiënten met een vermoedelijke TBLN.

Het maximale voordeel van Xpert MTB/RIF-testen kan worden verkregen door het gericht testen van patiënten met een vermoedelijke TBLN of tuberculose-meningitis. Toekomstig onderzoek moet zich richten op het evalueren van de mogelijke impact buiten de diagnostische nauwkeurigheid, zoals de behandelingsresultaten van de patiënt, de kosteneffectiviteit, haalbaarheid van schaalvergroting en algemene effecten van programmatische implementaties. Het werd duidelijk dat geen enkele vorm van microscopie-negatieve tbc of EPTB kan vertrouwen op een enkele diagnostische test. Elke gekozen diagnostische methode of aanpak moet gebaseerd zijn op een grondige beoordeling van het lokale tbc-opsporingssysteem en zijn lacunes. Er is een grote wereldwijde inspanning nodig om een eenvoudige, snelle en meer gevoelige diagnostische test voor tbc te ontwikkelen die gemakkelijk kan worden geïmplementeerd in lage-inkomst landen.

Chapter one: General introduction

1.1. Epidemiology of tuberculosis

1.1.1. Etiology, transmission and clinical manifestation

Tuberculosis (TB) is a chronic infectious disease caused by the *Mycobacterium tuberculosis* complex (MTBc); a group of mycobacteria which are fairly large, rod-shaped, aerobic, acid fast, and facultative intracellular parasites [1]. Mycobacteria are slow-growing micro-organisms with a generation time of 15-24 hours. Mycobacteria have a cell wall that is rich in lipids. This accounts for the difficulty in staining them with conventional techniques. The MTBc comprises closely related species responsible for strictly human or zoonotic TB. The complex consists of seven species including *M. tuberculosis, M. bovis*, *M. africanum, M. canetti, M. pinnipedii, M. microti,* and *M. caprae* [2]. The majority of TB in humans is caused by *M. tuberculosis* with a small proportion of TB disease caused by *M. bovis* and *M. africanum* [3].

TB is mainly transmitted from human to human by inhalation of the contagious, infectious droplets containing TB organisms that are expelled when a person with pulmonary TB sneezes, coughs, speaks or sings [4]. The inhaled bacilli lodge in the lung terminal air spaces and replicate within host macrophages. Less than 10% of persons infected develop symptoms and signs of active disease over a lifetime. The majority of immunocompetent individuals either eliminates *M. tuberculosis* or contains it in a latent state in which an equilibrium is established between host and pathogen, which is called latent TB infection (LTBI) [5]. Importantly, reactivation of LTBI can occur at any time in the infected individual's lifetime, depending on the waning of immunity due to chronic diseases such as diabetes, alcoholic liver disease, HIV coinfection, and use of steroids or other immunosuppressive drugs [6, 7].

TB can affect any organ in the body. The most common clinical manifestation of TB is pulmonary distress which is insidious onset. The clinical presentation of TB depends on the site of infection, the organ affected and its severity. Patients with pulmonary TB present with pulmonary symptoms (like productive cough, haemoptysis, chest pain and shortness of breath) and constitutional symptoms (like fever, poor appetite, weight loss, night sweats and anorexia) [8]. Uncontrolled cytokine release is responsible for many of the symptoms and signs of TB such as fever and wasting.

TB that affects any organ outside the pulmonary parenchyma is designated as extrapulmonary tuberculosis (EPTB). In particular, there are tissues where EPTB localizes more frequently: superficial and deep lymph nodes, pleura, bone and joints, central nerve system (CNS), abdomen and genitourinary TB [9, 10]. A small number of cases (0–3%) present with disseminated (miliary) TB [11]. In general, lymph node and pleural TB are the commonest forms of EPTB [9, 10]. All extra-pulmonary manifestations are increased in frequency among immunocompromised individuals such as HIV positive patients [12]. EPTB can also show similar symptoms as that of pulmonary TB like fever, night sweats, fatigue, loss of appetite and weight loss. In addition, patients often develop complaints specific to the body site affected [13, 14]. In general it has been estimated that 20%-50% of EPTB cases have concurrent pulmonary TB disease [15].

Lymph node TB is the most common form of EPTB at a worldwide level, accounting for approximately 35% of EPTB cases [16]. However in Ethiopia, the proportion of lymph node TB is exceptionally high accounting for more than 80% of EPTB cases [17]. The commonest clinical presentation is cervical lymphadenitis (60–90% of lymph node TB cases); other common sites of TB disease are mediastinal, axillary, mesenteric, perihepatic and inguinal lymph nodes. The localization of TB to the lymphatic system reflects a systemic involvement of TB infection, since *M. tuberculosis* bacilli follow the lymphatic drainage routes from a primary complex to the systemic lymphatic circulation [17]. The main complications of lymph node TB are fistulization and rupture, compression of adjacent structures, secondary bacterial infection and local extension of TB infection to the skin or to other organs. The differential diagnosis is wide and includes infections, neoplasms, non-specific reactive lymph node hyperplasia, sarcoidosis and connective tissue diseases [18, 19].

Bovine tuberculosis (BTB) is caused by *M. bovis*, a mycobacterium highly similar to *M. tuberculosis*, with both bacteria included in the *M. tuberculosis* complex. The main host of *M. bovis* is cattle but it also affects many other mammas including man. In humans, it is the most frequent cause of zoonotic TB which is clinically indistinguishable from TB caused by *M. tuberculosis* [20]. It has been estimated that *M. bovis* accounts globally for 3.1% of all human TB cases (2.1% of all pulmonary and 9.4% of all EPTB cases) [21]. In Africa, the extent of *M. bovis* involvement in the total TB burden is still largely unknown. This can be partly explained by the fact that most laboratories in sub-Saharan Africa do not have the capability to

differentiate *M. bovis* from *M. tuberculosis* [22]. BTB can be spread to humans by inhalation of bacteria-containing dust-particles and aerosols shed by infected animals or by ingestion of contaminated animal products (e.g. raw milk) [21].

1.1.2. Global burden of tuberculosis

TB has existed for millennia and remains a major global health problem, despite efforts and interventions for several decades. TB is one of the top 10 causes of death worldwide, and caused more deaths than HIV/AIDS in 2015 [23]. In 2015, there were 10.4 million new TB cases, of which 5.9 million were among men, 3.5 million among women and 1 million among children. It was estimated that 1.4 million people died from TB among HIV-negative people and an additional 0.39 million deaths from TB among HIV-positive people in 2015. Globally in 2015, an estimated 3.9% (95% CI: 2.7–5.1%) of new cases and 21% (95% CI: 15–28%) of previously treated cases had multidrug-resistant TB or rifampicin-resistant TB (MDR/RR-TB). The WHO estimates that 480,000 cases of MDR-TB occurred globally in 2015, and that at least 10% of those cases were XDR-TB or totally drug-resistant TB [23].

Most of the estimated number of TB cases in 2015 occurred in Asia (61%) and the WHO African Region (26%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (3%) and the Region of the Americas (3%). The 30 high TB burden countries accounted for 87% of all estimated incident cases worldwide. The six countries that stood out as having the largest number of incident cases in 2015 were in descending order India, Indonesia, China, Nigeria, Pakistan and South Africa, accounting combined for 80% of the global total. An estimated 11% (range, 9–14%) of the incident TB cases in 2015 were among people living with HIV. The proportion of TB cases coinfected with HIV was highest in countries in the WHO African Region, and exceeded 50% in parts of Southern Africa [23].

TB rates have been falling in all six of the WHO regions. Globally, the average rate of decline in the TB incidence rate was 1.4% per year in 2000–2015, and 1.5% between 2014 and 2015. This needs to accelerate to 4–5% per year by 2020 to achieve the milestones for reductions in cases and deaths set in the End TB Strategy [24]. In Africa, the slower decline in TB rate could be due to multiple factors, such as widespread poverty, the HIV epidemic, poor socioeconomic status, limited laboratory capacity for case detection, treatment barriers, inadequate living conditions and the emergence of drug-resistance [25, 26].

1.1.3. Tuberculosis situation in Ethiopia

TB is among the leading causes of morbidity and mortality in Ethiopia. Ethiopia is among the 30 high-burden countries for TB, TB/HIV and multidrug-resistant TB (MDR-TB) in the world. In 2014, WHO estimated that there were 200,000 new TB cases in Ethiopia, ranking the country 10th among the world's 22 high-burden countries for TB, and 4th in Sub-Saharan Africa [23, 27]. While TB kills an estimated 32,000 Ethiopians every year (more than 80 people per day), it also has a long-term impact on the economy of Ethiopia's population. Since the majority of TB cases occur among young adults and children, TB cumulatively exacts a heavy economic cost, impeding the country's drive toward becoming a middle-income country. **Table 1** shows the latest estimates of tuberculosis in Ethiopia for 2015 [23].

According to the first Ethiopian national population based TB prevalence survey in 2010/2011, the prevalence of bacteriologically confirmed TB cases was 277/100,000 [28]. The proportion of PTB cases detected is only 60-65%. Among the PTB cases, the number of smear-negative cases exceeds that of smear-positive. The prevalence survey showed that smear- positive cases accounted for only 43% of culture-positive cases [28]. Moreover, the incidence of EPTB among TB patients in Ethiopia has steadily increased since the 1990s, reaching an average of around 37% across the country [29]. EPTB comprises up to 40% of all TB cases registered annually in Ethiopia compared to 15% worldwide. More than 80% of all EPTB cases involve lymph node TB that currently accounts for around 30% of all incident TB cases in the country [30]. This is a peculiar picture seen in Ethiopia for over a decade. Nevertheless, the components of the Ethiopian National Tuberculosis Control Program, including diagnosis, directly observed treatment practices, and patient follow-up and support, are mainly focused on the less frequent smear positive pulmonary TB.

Ethiopia also ranks 15th among the 27 countries with high burden of MDR-TB [14]. MDR-TB is deadlier, harder to diagnose and treat than (pan-) susceptible TB. According to a recent unpublished study, the proportion of MDR-TB among new and previously treated TB cases in Ethiopia was 2.3% and 17.6% respectively [31]. Based on the latest WHO estimates [23], only about 39% of Ethiopia's MDR-TB patients were being diagnosed as having resistance, indicating that the majority of the expected MDR-TB cases in Ethiopia remain undiagnosed, are treated with ineffective rifampicin based regimens, and continue to transmit the disease in the community.

Table 1: Latest estimates of tuberculosis in Ethiopia, 2015

Population		99 million
Global rank of TB burden countries		10th
Estimates of TB burden, 2015		
	Number (thousands)	**Rate (per 100 000 population)**
Mortality (excludes HIV+TB)	25 (15–38)	26 (15–38)
Mortality (HIV+TB only)	3.9 (1.6–7.3)	4 (1.6–7.4)
Incidence (includes HIV+TB)	191 (141–249)	192 (142–250)
Incidence (HIV+TB only)	16 (10–23)	16 (10–23)
Incidence (MDR/RR-TB)	6.2 (3.5–8.9)	6.2 (3.5–9)
TB case notifications, 2015		
Total cases notified		137 960
Total new and relapse		135 951
• % tested with rapid diagnostics at time of diagnosis		6%
• % with known HIV status		77%
• % pulmonary		70%
• % bacteriologically confirmed among pulmonary		54%
Drug-resistant TB care, 2015		
	New cases	**Previously treated cases**	**Total number**
Estimated MDR/RR-TB cases among notified pulmonary TB cases			3300(2100-4600)
Estimated % of TB cases with MDR/RR-TB	2.7% (1.5–4)	14% (5.6–23)	
% notified tested for rifampicin resistance	9%	75%	24073
MDR/RR-TB cases tested for resistance to second-line drugs			113
Drug-resistant TB care, 2015		
	MDR/RR-TB	**XDR-TB**
Laboratory-confirmed cases	597	2
Patients started on treatment	597	2
Treatment success rate and cohort size		
	Success	**Cohort size**
New cases registered in 2014	89%	121 563
MDR/RR-TB cases started on second-line treatment in 2013	68%	397

Source: WHO. Global Tuberculosis Report, 2016 [23]. MDR-TB=multidrug resistant TB, RR-TB=rifampicin resistant TB, XDT=extensively drug resistant TB

1.1.4. Tuberculosis control in Ethiopia

Ethiopia has adopted the WHO recommended directly observed therapy, short course (DOTS) strategy in 1992. The program is combined and implemented with the leprosy program; named National Tuberculosis and Leprosy Control Program since 1994. Since then TB control efforts have been decentralized to public health facilities (hospitals, health centers and health stations) [32].The national TB control program (NTCP) is organized in a hierarchical fashion with varying responsibilities under the Federal Ministry of Health (FMOH). Within an integrated health system, the program relies on supervisory staff at the national, regional, zonal and district levels, which has basic knowledge and skill on TB, TB/HIV and leprosy.

The NTCP has currently achieved 100% geographical coverage and more than 95% of public hospitals and health centers offer DOTS [33]. The TB program emphasizes the need to increase the access of quality DOTS by expanding TB diagnostic and treatment services in line with the increasing number of public and private health facilities. The program is scaling up the access to drug sensitivity testing (DST) and MDR-TB treatment with quality-assured drugs supplied through the Green Light Committee. To maximize case detection and treatment, the TB program engages all health care providers in the country (e.g. private health facilities and health extension workers) through the massive expansion of diagnosis and treatment services [28, 34]. Considerable progress has been achieved in numbers of health facilities providing TB services: 156 government hospitals, 3, 335 public health centers, 48 private hospitals, 222 private health facilities, 7 primary clinics, and 10, 013 health posts. In terms of laboratory capacity, Ethiopia has 2,986 health facilities with laboratories providing quality-assured microscopy for TB diagnosis. Smear microscopy, which is the most commonly used tool in Ethiopia and most high TB-burden countries, identifies only half of TB cases that could be diagnosed by culture, resulting in a greater reliance on clinical diagnosis. Recently, there are about nine laboratories capable of performing TB culture and drug susceptibility testing in Ethiopia [35]. Nevertheless, of the total cases notified only 6% were diagnosed by rapid diagnostic tests at the time of diagnosis (**Table 1**). Though the case detection rate of drug resistant TB is gradually increasing as shown in Figure 1 from 2009-2013, the detection rate remains very low, less than 20% of expected drug resistant TB cases were detected as having resistance.

In 2004, Ethiopia introduced an innovative community-based strategy, the Health Extension Program (HEP) to deliver preventive and promotive health services as well as selected high-impact curative interventions at community level. The program aims to improve the utilization of health services by bridging the gap between the community and health facilities through the deployment of health extension workers (HEWs). With the decentralization of TB care, TB treatment follow-up services were integrated into the HEP. This allows TB patients to take their daily medicine at home or at the health post under the direct observation of the HEWs and TB treatment supporters. The HEWs are oriented in how to provide DOTS and support the patient during the treatment. Despite Ethiopia's significant commitment in reducing TB disease and death rates, the government recognizes the current burden of TB, and acknowledges the need for new approaches to control the spread of both drug-sensitive and drug-resistant TB [35].

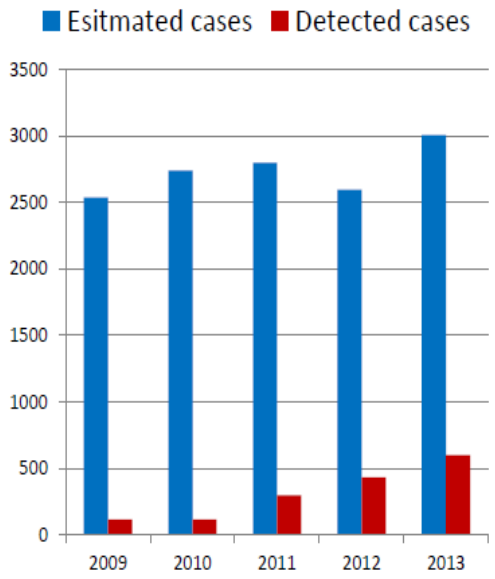

Figure 1: Estimated versus detected MDR-TB cases among notified TB cases in Ethiopia: 2009-2013.
Source: *GLI/GDI meeting, Geneva, April 2015*

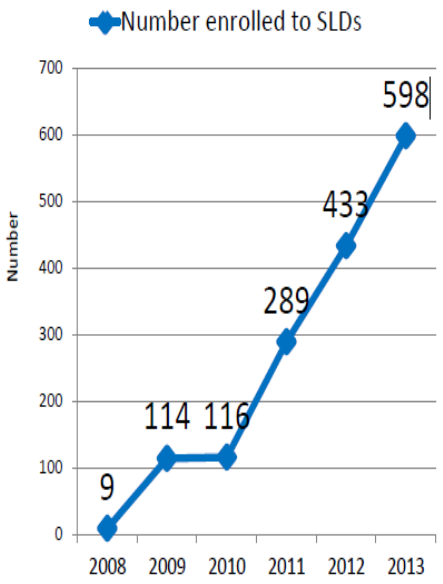

Figure 2: MDR-TB patients enrolled to second line drugs in Ethiopia: June 2009-July 2014. SLDs- second line drugs
Source: *GLI/GDI meeting, Geneva, April 2015*

1.2. Diagnosis of tuberculosis

Early detection of the cases and prompt treatment are crucial for TB control. TB diagnosis mainly depends on the clinical presentation of the disease and identification of the bacilli in clinical samples. Many TB diagnostic tests are available although no single diagnostic test for TB exists that can be performed rapidly, simply, inexpensively, and accurately as a stand-alone-test. TB can be diagnosed by a variety of techniques: chest X-ray, smear microscopy, culture, nucleic acid amplification tests and immunological methods [36].

1.2.1. Smear microscopy

Smear microscopy is the oldest and still the most widely used method for detection of TB in low and middle income countries [37, 38]. Two methods are available for the direct examination of the bacilli in clinical samples: conventional staining with carbol-fuchsin [Ziehl-Neelsen (ZN)] and auramine-based staining. Both techniques rely on the fact that the cell wall of mycobacteria is rich in complex lipids and when stained with carbol-fuchsin or fluorochromes under special staining conditions is not easily decolorized. Since these cell wall-dye complexes are resistant to destaining with alcohol-acid solutions, mycobacteria are referred to as “acid-fast bacilli” or “AFB” [39].

The conventional smear microscopy (ZN) is simple, rapid, inexpensive and efficient in detecting those cases of pulmonary TB that are most infectious [38]. However, the test has a low sensitivity with a detection limit of between 5,000–10,000 bacilli/ml of sample [40, 41, 42]. The overall clinical sensitivity of sputum AFB smear is 20–70% depending on the burden of mycobacteria, the type of AFB stain used, and experience of the laboratory technician, while the positive predictive value for mycobacteria is high (> 95%) [43]. For patients with a low bacillary load, such as HIV patients, infants and patients with EPTB, true positive cases can easily be missed [39, 44]. Smear microscopy does not allow for mycobacterial species identification, nor does it give an indication of the viability of mycobacteria in the sample, or the resistance profile of the bacilli. Many reports have suggested centrifugation and overnight sedimentation, preceded with any of several chemical methods (including bleach) is slightly more sensitive (6-9%) than direct smear microscopy, while specificity may be slightly decreased (1-3%) by sputum processing methods [45, 46].

Methods which apply a fluorochrome have been used to stain AFB for many years. Using this method, mycobacteria are detected as bright fluorescent rods against a darker background under fluorescence microscopy [47]. In general, fluorescence methods are on average 10% more sensitive than conventional smear microscopy (ZN stain). Because fluorochrome-stained smears are screened at lower magnifications, it takes less time to examine these smears than to examine smears stained with ZN stain and still results in a higher sensitivity and a similar specificity. Light-emitting diode (LED) is primarily developed to provide resource-limited settings with access to the benefits of fluorescent microscopy. LED microscopes are less expensive, the bulbs have a long half-life and do not pose the risk of releasing potentially toxic products, and are reported to perform equally well in a light room. For these reasons, the WHO has endorsed the global phase out of conventional ZN light microscopy in favor of auramine AFB staining using fluorescent LED microscopy [48].

Following the WHO recommendation, the National Tuberculosis Control Program has started to roll-out LED-fluorescence microscopy in Ethiopia. The uptake was generally enthusiastic and few difficulties were encountered during the adoption of the fluorescence microscopy. The main challenges during the implementation were due to artifacts being interpreted as AFBs and negative slides therefore being called positive. Due to this and some technical problems, LED-fluorescence microscopy is not yet routinely used in all TB diagnostic centers in Ethiopia [49].

1.2.2. Mycobacterial culture

Detection of AFB directly in patient specimens by smear microscopy is a rapid and cheap method. However, it requires a high bacterial count in the specimen for a reliable result. Hence, there is a need to culture mycobacteria in special liquid and solid media, a method which is still the gold standard for detecting mycobacteria in different specimens. The primary advantage of culture tests over sputum microscopy is their higher sensitivity, allowing for the detection of very low numbers of bacilli (approximately 10-100 bacilli/ml of sputum) [50]. Culture tests are also used for the detection of treatment failures and for diagnosing EPTB. The use of culture can increase the number of TB cases found by 30–50%. Moreover, cultures are used for species identification and drug susceptibility testing (DST) [51, 52]. Culturing mycobacteria is however time consuming and can take up to eight weeks as TB bacilli and

many other mycobacteria grow very slowly. In addition, culture requires technical skills and a high complexity laboratory to meet biosafety standards.

Culture is traditionally performed on solid egg-based media, such as Löwenstein-Jensen (L-J), which is composed of egg suspension, potato flour, salts, and glycerol. Egg suspension provides fatty acids and protein required for the metabolism of mycobacteria. When heated, the egg albumin coagulates, thus providing a solid surface for inoculation. L-J medium also contains malachite green as an inhibitor of non-mycobacterial organism, especially for sputum culture. L-J containing glycerol favors *M. tuberculosis* growth, while L-J without glycerol but containing sodium pyruvate enhances *M. bovis* growth [1, 52]. While some laboratories still use L-J media, many have transitioned to using more chemically defined agar-based media optimized for faster mycobacterial growth. Agar-based media contains Middlebrook and oleic acid-albumin-dextrose-catalase (OADC) enrichment and allows for visible MTBc colony growth in 7–12 days as compared to 18–24 days with L-J media. Because of the transparency of Middlebrook plates, *M. tuberculosis* micro colonies with typical cord formation can be detected and counted using a microscope. Moreover, visibility of colonial morphology on agar plates is better than on egg-containing slants, aiding the identification of mycobacteria. A disadvantage of Middlebrook media is that the surface dries more rapidly than egg-based media [53].

In 1980, BACTEC 460 TB detection system was introduced commercially for mycobacterial recovery from clinical specimens and drug susceptibility testing. The BACTEC 460 TB System has been reported to yield 15-20% increased culture positivity of clinical specimens as compared to conventional solid media such as L-J medium, with an average time-to-detection of positive growth from 8 to 14 days as compared to 3 to 5 weeks on solid media [54, 55, 56]. The high efficiency of the BACTEC TB System is due to the use of liquid medium, the presence of a growth enhancing substance and detection of metabolic activity rather than observation of mature colonies. One of the disadvantages of the BACTEC 460 TB System is the use of ^{14}C-Labeled radioactive substrate [55].

The Becton Dickinson Company (BD) developed a new system called Mycobacteria Growth Indicator Tube (MGIT™), which is non-radiometric and offers the same rapid, sensitive and reliable methods of testing as the BACTEC 460 TB System. BACTEC MGIT 960 (MGIT 960) is the fully automatic system for detection of mycobacterial growth and drug susceptibility testing of

MTBc. The BACTEC MGIT 960 system involves culture tubes containing a fluorescent sensor that detects the concentration of oxygen in the medium. Numerous studies have evaluated the MGIT system for primary isolation of mycobacteria and DST as compared with L-J media and BACTEC 460 [57, 58, 59]. Better performance of MGIT960, as compared with other commercially available TB liquid culture and molecular amplification systems, has also been reported [60, 61].

1.2.3. Nucleic-acid amplification tests (NAAT)

Nucleic acid amplification tests (NAATs) are molecular systems that can detect small quantities of genetic material (DNA or RNA) from microorganisms, such as *M. tuberculosis*. A variety of molecular amplification methods are available, of which PCR is the most common. Developed from in-house techniques to fully automated assays, NAAT may provide results in a couple of days or even hours [62]. Molecular methods support culture either by serving as a rapid direct test on specimens or by enabling a rapid and unequivocal species differentiation from culture material. NAA testing provides the possibility for earlier laboratory diagnosis of TB as compared to traditional methods, leading to earlier initiation of treatment, use of appropriate isolation precautions to interrupt transmission, and improved patient outcomes [63, 64].

The sensitivity of NAATs to detect TB is high (95%) in smear-positive sputum samples, with specificity of 90-100% [65].The sensitivity of many NAAT is greatly reduced when testing smear-negative sputum specimens. Despite the reduction in sensitivity when testing AFB smear-negative, NAATs are still recommended for patients with suspected TB due to superior performance as compared to AFB staining. However TB disease cannot be ruled out even from a smear-negative specimen with a negative NAAT result [66]. In addition, NAATs cannot differentiate between live and non-viable MTBc, so they cannot be used to monitor response to treatment. However, a few studies reported pre-treatment of sputum samples with propidium monoazide (PMA), which can selectively amplify DNA derived from viable *M. tuberculosis* in clinical specimens. This approach could be useful for treatment monitoring in pulmonary TB patients [67]. Studies also showed that molecular bacterial load assay, a culture-free biomarker detecting *M. tuberculosis* 16S rRNA, can be used for rapid and accurate quantification of sputum *M. tuberculosis* bacillary load during treatment [68]. NAATs rely upon appropriate laboratory infrastructure to house delicate equipment and the reagents are often

cold chain dependent. The complexity of non- or partially automated NAATs requires highly skilled technicians [69].

1.2.3.1. GeneXpert MTB/RIF assay

GeneXpert MTB/RIF assay (Cepheid, Sunnyvale CA, USA; hereafter called Xpert MTB/RIF) is an automated molecular test that makes use of the molecular beacons to identify DNA sequences amplified in a hemi-nested real time-PCR to detect the presence of MTBc and simultaneously of rifampicin resistance in clinical specimen in less than two hours. Five overlapping nucleic acid hybridization probes, labeled with coloured fluorophores, are used for binding to an 81-basepiar core region of the wild type *rpoB* gene. The Xpert MTB/RIF diagnostic system integrates and automates sample processing, nucleic acid amplification, and detection of the target sequences in samples using semi-nested, real-time PCR [70]. The only manual step is the addition of a bactericidal buffer to the sputum specimen before transferring a defined volume to the cartridge. The cartridges are pre-loaded with all reagents necessary for sample processing, DNA extraction, amplification, and laser detection of target amplicon binding to the molecular beacons. The assay was designed to be robust, easy and safe to use in microscopy laboratories where biological safety cabinets are usually not available [71, 72, 73].

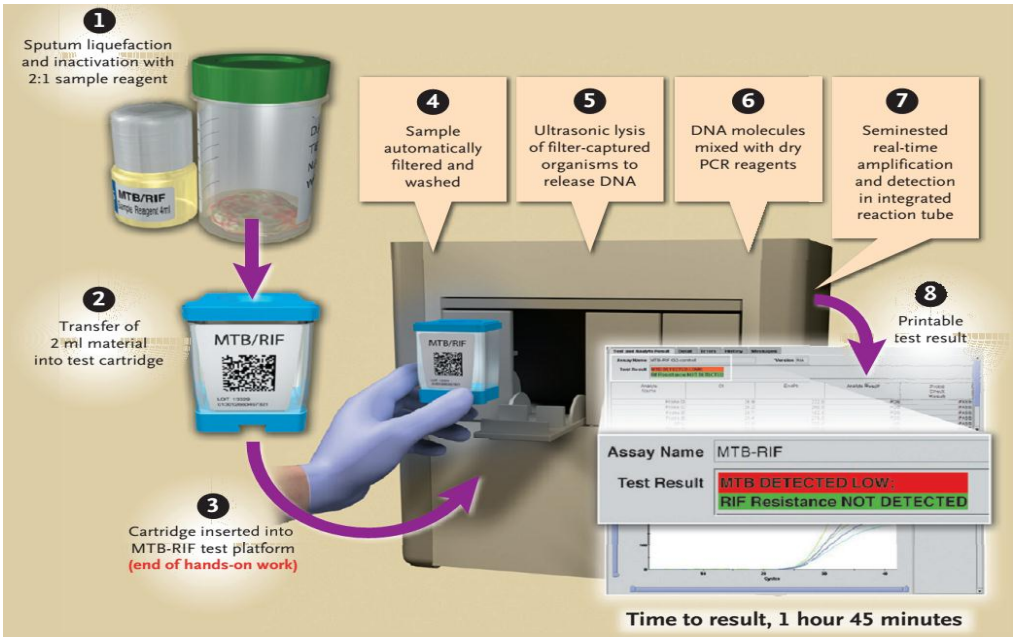

Figure 3: Xpert MTB/RIF assay sample preparation and testing process. Figure taken from Boehme, *et al. NEJM* 2010 [74]

Results from analytical studies showed that the Xpert MTB/RIF has analytic sensitivity for five genome copies of purified DNA, and 131 colony forming units/ml of *M. tuberculosis* spiked into sputum [72]. A meta-analysis involving 15 published studies on PTB reported an overall pooled sensitivity of 90.4% (95% CI: 89.2–91.4) and a pooled specificity of 98.4% (98.0–98.7). The pooled sensitivities for sputum smear-negative and smear-positive TB disease were 75.0% and 98.7%, respectively [75]. The WHO Expert Group Review found that single Xpert MTB/RIF test detected 91% of culture-confirmed TB patients (99% smear-positive and 80% smear-negative). Rifampicin resistance was detected with 95.1% sensitivity and 98.4% specificity although specificity is higher when adopting the full *rpoB* sequence as gold standard [124]. Time to detection was less than 1 day with Xpert MTB/RIF compared to 17 days (liquid culture); greater than 30 days (solid culture); and greater than 75 days (phenotypic DST). Smear-negative TB patients started treatment after 4 days when Xpert MTB/RIF was used compared to 58 days when Xpert MTB/RIF not used. Although HIV co-infection substantially decreased the sensitivity of microscopy, it did not significantly affect the performance of Xpert MTB/RIF [76].

WHO confirmed a solid evidence base to support widespread use of Xpert MTB/RIF for detection of TB and rifampicin resistance [76] and forwarded the following recommendations in 2011 (with update in 2013):

- Xpert MTB/RIF should be used rather than conventional microscopy, culture and DST as the initial diagnostic test in adults suspected of having MDR-TB or HIV-associated TB (strong recommendation, high-quality evidence).
- Xpert MTB/RIF should be used rather than conventional microscopy, culture and DST as the initial diagnostic test in children suspected of having MDR-TB or HIV-associated TB (strong recommendation, very low-quality evidence).
- Xpert MTB/RIF may be used rather than conventional microscopy and culture as the initial diagnostic test in all adults suspected of having TB (conditional recommendation acknowledging resource implications, high-quality evidence).
- Xpert MTB/RIF may be used rather than conventional microscopy and culture as the initial diagnostic test in all children suspected of having TB (conditional recommendation acknowledging resource implications, very low-quality evidence).
- Xpert MTB/RIF may be used as a follow-on test to microscopy in adults suspected of having TB who are not at risk of MDR-TB or HIV-associated TB, especially when further

testing of smear-negative specimens is necessary (conditional recommendation acknowledging resource implications, high-quality evidence).

1.2.3.1.1. Xpert MTB/RIF for diagnosing EPTB

The Xpert MTB/RIF assay was initially developed, optimized, and endorsed for the detection of pulmonary TB using sputum [71, 77]. More recently, assessments of the Xpert MTB/RIF test have extended to various non-respiratory clinical samples from patients with EPTB [78, 79, 80]. Investigation for use in EPTB is far more complex because of the diversity of clinical sample types, difficulties in obtaining adequate tissue for analyses, the challenge of providing a rigorous reference standard for comparison, and the range of ways to process samples before analysis. To establish the diagnosis of EPTB, appropriate specimens including fine needle aspirate of lymph node, pleural fluid, pericardial, peritoneal fluid, cerebrospinal fluid, blood, urine, or biopsy/tissue specimens should be obtained for Xpert MTB/RIF testing [78, 79].

The reported sensitivity of Xpert MTB/RIF for EPTB was highly heterogeneous between sample types, ranging from 25.0% to 96.6% [75, 81, 82]. Using culture as reference standard, the pooled sensitivities exceeded 70% for tissue biopsy samples, fine needle aspirates, pus samples, cerebrospinal fluid (CSF), and gastric aspirates [81, 82, 83]. Lower sensitivity has been noted when testing pleural, pericardial, peritoneal, and synovial fluid samples [82, 84]. Increasing evidence from diagnostic accuracy studies might, in the future, open the possibility for international recommendations for use of the assay for diagnosis of EPTB.

The specificity of Xpert MTB/RIF was very high across the majority of studies, high-lighting its utility as a rule-in test for TB diagnosis that can be used to reliably inform the start of TB treatment when positive. In contrast, sensitivity was extremely heterogeneous, with much higher sensitivity being typically seen when testing lymph node samples, other tissue samples and cerebrospinal fluid as compared to the results of testing pleural fluid and other serous fluids [85]. Based on a systematic review by Denkinger and colleagues, the WHO recommends Xpert MTB/RIF over conventional tests for diagnosis of TB in lymph nodes and other tissues, and as the preferred initial test for diagnosis of TB meningitis [83]. Though, WHO has recommended Xpert MTB/RIF for diagnosis of EPTB in cerebrospinal fluid, lymph node and other tissues, the quality of evidence is very low. WHO has forwarded the following recommendations on the use of Xpert MTB/RIF for diagnosis of EPTB:

- Cerebrospinal Fluid (CSF): Xpert MTB/RIF should be used in preference to conventional microscopy and culture as the initial diagnostic test in testing CSF specimens from patients presumed to have TB meningitis (strong recommendation given the urgency of rapid diagnosis, very low quality of evidence)
- Lymph node and other tissues: Xpert MTB/RIF may be used as a replacement test for usual practice (including conventional microscopy, culture, and/or histopathology) for testing of specific non-respiratory specimens (lymph nodes or other tissue) from patients presumed to have EPTB (conditional recommendation, very low quality of evidence).

1.2.3.1.2. Implementation of Xpert MTB/RIF in Ethiopia

WHO has recommended starting roll-out of the Xpert MTB/RIF system in countries to improve TB diagnosis and detection of rifampicin resistance. The Ethiopian National TB Control Program has developed an implementation guideline on the use of Xpert MTB/RIF in Ethiopia. The purpose of this guideline is to describe and facilitate processes for the smooth introduction of the Xpert MTB/RIF assay system into the National TB diagnostic network. In line with the WHO recommendations and taking the burden of TB/MDR TB in Ethiopia, it is recommended that the Xpert MTB/RIF test can be used in the following clinical conditions [86]:

I. Diagnosis of TB and MDR-TB in presumptive MDR-TB cases: Xpert used as primary test:
   - Failure of previously treated cases (smear positive at or after 5 months)
   - Symptomatic contacts of MDR-TB cases
   - Failure of new cases (smear positive at or after 5 months)
   - Previously treated cases (return after relapse, return after Loss To Follow Up)
   - Sputum Smear positive at 3 months while on treatment,
   - Symptomatic individuals from known high risk groups (Example HCWs)
   - Vulnerable groups (e.g. prisoners, homeless, refugees, migrants)

II. Diagnosis of TB in HIV positive Presumptive TB cases: Xpert used as primary test.
   - HIV positive Presumptive TB clients
   - EPTB Presumptive TB individuals (samples like CSF, LN aspirates, Biopsy, pus)

III. Diagnosis of TB and MDR-TB in Children with Presumptive TB: Xpert as primary test:
   - Children under 14 year of age- all presumptive TB cases (Sputum or other samples like gastric aspirate, induced sputum, LN aspirate, Pus, CSF, Biopsy can be tested)

Xpert MTB/RIF is undergoing phased implementation in Ethiopia for routine diagnosis of TB and drug resistant TB. As of March 2015, about 100 Xpert MTB/RIF machines were distributed across the country (**Figure 4**). The ministry of health has a plan to scale-up the implementation of Xpert MTB/RIF throughout the laboratories for the diagnosis of TB and screening of drug resistance TB. Rollout of Xpert MTB/RIF should be addressed in a systematic and coordinated approach to optimize the usefulness of the technology under routine program conditions and to ensure maximum efficiency. In addition, country-specific adaptation of the diagnostic algorithms (e.g., prioritization of patient groups to be tested) may be dictated by the availability of resources. WHO has therefore recommended an on-going operational research to refine and inform future policy.

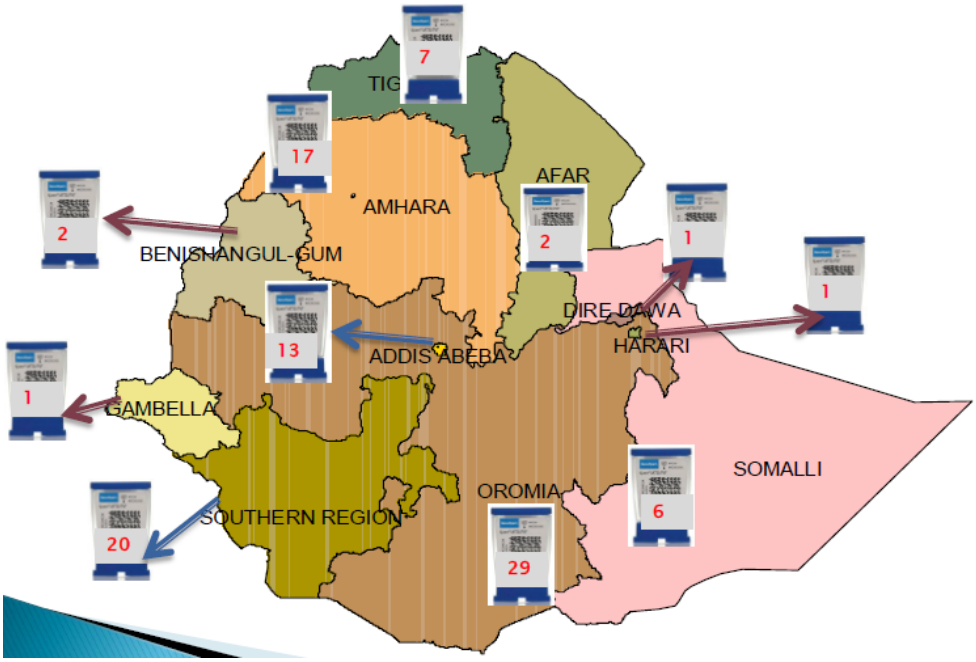

Figure 4: GeneXpert MTB/RIF machine distribution in the different region of Ethiopia as of March 2015.
Source: *GLI/GDI meeting, Geneva, April 2015*

1.2.4. Serological tests

Diagnostic tests for active TB based on the detection of antibodies (serological tests) have been commercially available for decades. In comparison to smear microscopy and culture, serological tests take only minutes to perform. These technologies are very attractive candidates for the simple, inexpensive, and, ideally, point-of-care (POC) diagnosis of infectious

diseases. However, attempts to successfully develop sensitive and specific serological tests for the diagnosis of TB have been ongoing for decades, without a major breakthrough [87].

A systematic review of commercial serological tests for the diagnosis of TB suggests inconsistent and imprecise estimates of sensitivity and specificity [88]. Overall, commercial tests varied widely in sensitivity (10% to 90%) and specificity (47% to 100%). Accuracy was higher in smear-positive than in smear-negative specimens, and specificity was higher in healthy populations than in patients for whom TB disease was initially suspected and subsequently ruled out. There were insufficient data to determine the accuracy of most commercial tests in smear-negative patients, and none of the assays performed well enough to replace AFB smear microscopy [87]. Overall, quality of evidence for serological test was graded very low for studies involving pulmonary and EPTB cases. WHO has given a negative advice on serological tests for TB [89].

1.2.5. Interferon gamma release assays (IGRAs)

Interferon gamma (IFN-γ) release assays (IGRAs) have been introduced as an alternative to the tuberculin skin test for the diagnosis of LTBI [90]. IGRA tests measure the interferon gamma release from T cells after stimulation by *M. tuberculosis*- specific antigens via an enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunospot (ELISPOT) assay. Both, ELISA and ELISPOT, assays are unable to differentiate between active TB and LTBI when used on peripheral blood [91, 92, 93]. However, IGRAs may have a use in the diagnosis of active TB if disease site-specific lymphocytes are used. MTB-specific T cells are present at a much higher frequency at the site of disease compared with the periphery [93]. MTB-specific T cells have been shown to be concentrated in samples such as CSF, ascites, and pleural effusion from individuals with EPTB [92, 94, 95]. Patel *et al* [96] have reported that the CSF-ELISPOT platform can act as a rapid rule-in test for TB meningitis in a TB-endemic region with high HIV prevalence when used in combination with simple tests to rule out bacterial infection and cryptococcal meningitis (sensitivity, 82%; specificity, 100%). Two studies [97, 98] reported that pleural fluid IFN-γ determination is a sensitive and specific test for the diagnosis of TB pleuritis. In high burden countries like Ethiopia, IGRAs have limited value as diagnostic tools to screen and rule out PTB or EPTB [99, 100]. Therefore, the potential use of these assays in the clinical routine awaits further confirmatory studies, especially in high-incidence, resource-poor settings.

1.2.6. Lateral flow-urine lipoarabinomannan assay (LF-LAM)

Mycobacterial antigen detection is an attractive option for TB diagnosis, overcoming many of the limitations inherent in serological antibody tests. Compared with sputum and extra-pulmonary samples, urine is very practical as a clinical sample. It is easy to collect from both adults and children, quality is likely to be less variable, and it is safer to handle. Lipoarabinomannan (LAM) is a mycobacterial antigen that can be detected in urine samples of patients with active TB disease [101]. LAM is a heat stable glycolipid specific to mycobacteria that is released by metabolically active or degrading mycobacteria, filtered by the kidney, and found in the urine of patients with TB [102]. LAM is an attractive diagnostic target as urine processing requires limited infection control measures, presence of LAM in urine is indirectly related to human immune response, and its detection process is amenable to inexpensive POC platforms.

Studies have evaluated commercially available LF-LAM tests (Alere Determine™ TB LAM Ag, Alere Inc, Waltham, MA, USA) that detect LAM antigen in urine for the diagnosis of active TB [87, 103, 104]. For individual studies, sensitivity estimates ranged from 23% to 84%, and specificity estimates from 75% to 99%. The pooled sensitivity across studies was 44% (95% CI, 31- 60%) and the pooled specificity was 92% (95% CI, 83-96%). Using the composite reference standard, the individual sensitivity estimates ranged from 11% to 45% and specificity estimates ranged from 96% to 98%. The pooled sensitivity was 28% (95% CI, 13- 51%) and the pooled specificity was 97% (95% CI, 93-99%) [105]. Unlike traditional TB diagnostic methods, the LF-LAM test sensitivity was significantly higher in HIV-TB co-infection with further increases with lower CD4 counts. Several hypotheses may explain the higher sensitivity of urine LAM detection in patients with HIV-related immunosuppression, including higher bacillary burden and antigen load, greater likelihood of TB in the genitourinary tract, and greater glomerular permeability to allow increased antigen levels in urine. Urine-based TB diagnosis is definitively attractive, and further work is needed to improve the LAM assay.

Based on the available data, WHO forwarded the following policy recommendations for the use of the LF-LAM assay [105]:

- LF-LAM should be used for persons with HIV infection with low CD4 counts or who are seriously ill (strong recommendation, low quality of evidence).

- LF-LAM may be used to assist in the diagnosis of TB in HIV positive adult in-patients with signs and symptoms of TB (pulmonary and/or extrapulmonary) who have a CD4 cell count less than or equal to 100 cells/μL, or HIV positive patients who are seriously ill regardless of CD4 count or with unknown CD4 count (conditional recommendation; low quality of evidence).
- LF-LAM should not be used as a screening test for TB (strong recommendation, low quality of evidence).

1.3. Detection of drug resistant TB

MDR-TB poses daunting challenges due to the complex requirements for diagnosis and treatment [106]. The ability to rapidly and accurately detect drug resistance in *M. tuberculosis* clinical specimens is essential for appropriate treatment to be initiated in patients suffering from TB and for the prevention of further spread of drug-resistant strains. No single diagnostic test currently satisfies all the demands of "rapid", "affordable", and "easy". DST using solid media can take 6 weeks or more due to the slow growth of the MTBc organism [107]. The WHO has endorsed the use of commercially available liquid culture systems (BACTEC MGIT960) to rapidly detect MDR-TB, however, due to the tests' complexity and cost, as well as the need for sophisticated laboratory infrastructure and trained personnel, uptake has been limited in many resource-constrained settings [108]. Recent work has evaluated novel rapid direct tests in which decontaminated sputum specimens are inoculated directly into drug-free and drug-containing medium [109, 110]. Several alternative culture-based methods for TB detection and DST are available, including the microscopic observation drug susceptibility (MODS) and nitrate reductase assay (NRA) [111, 112]. WHO has endorsed MODS and the NRA for direct drug susceptibility testing of sputum specimens [113]. WHO also indicated that DST for second-line anti-TB agents should be done on all *M. tuberculosis* isolates with confirmed MDR/RR-TB to confirm or exclude extensively drug resistant TB (XDR-TB) [113].

Genotypic methods have considerable advantages in terms of scaling up the programmatic management and surveillance of drug-resistant TB, offering quicker diagnosis, standardized testing, the potential for high throughput, and having fewer requirements for ensuring laboratory biosafety. Resistance to the most common anti-TB drugs, and particularly to rifampicin, is amenable to rapid molecular detection because the vast majority of resistant strains contain mutations in restricted regions of specific genes [74]. There are two

commercially available line-probe assays endorsed by WHO [INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium) and GenoType® MTBDRplus assay (Hain LifeScience GmbH, Nehren, Germany)] [114, 115]. The INNOLiPA Rif.TB assay targets common mutations in the *rpoB* gene associated with rifampicin resistance while the GenoType® MTBDRplus assay also targets the common mutations in *katG* and *inhA* genes associated with isoniazid resistance in addition to the mutations in the *rpoB* gene. Advantages of line probe assays are that they can provide a result for detection of TB and drug resistance in 1 to 2 days, directly from specimens. Line probe assays have both high sensitivity (greater than 97%) and high specificity (greater than 99%) for the detection of rifampicin resistance alone, or in combination with isoniazid (sensitivity greater than 90%; specificity greater than 99%) [116]. WHO has also endorsed the use of molecular line probe assays for rapid DST of selected second-line drugs (such as fluoroquinolones and injectable second-line drugs) [117]. Drawbacks are that line probe assays are expensive and must be used in reference laboratories with appropriate pre- and post-PCR facilities [117, 118].

The Xpert MTB/RIF assay detects *M. tuberculosis* as well as mutations that confer rifampicin resistance [70, 71]. The molecular beacons that target the *rpoB* gene cover all the mutations found in the 81-bp core region. Rifampin resistance is also a predictor of MDR-TB since the majority of rifampin-resistant isolates will also be isoniazid-resistant [119, 120]. The pooled sensitivity and specificity of Xpert MTB/RIF for detection of rifampicin resistance were 94% (95% CI: 87% to 97%) and 98% (95%CI: 97% - 99%), respectively [121, 122]. Given the high negative predictive value (98%) of Xpert MTB/RIF in detecting rifampicin resistance, a negative result accurately excludes the possibility of rifampicin resistance and, usually, no further testing is required to confirm negative results. However, regional differences may occur due to the clonal spread of strains with mutation outside 81-bp region. A study done in Swaziland showed that 30% of rifampicin resistant *M. tuberculosis* strains have mutations at *rpoB* I491F which is outside 81-bp region of *rpoB* gene. This can substantially reduce the sensitivity of Xpert MTB/RIF–based diagnosis and presumably results in under-diagnosis and potentially inadequate treatment [123].

The specificity of Xpert MTB/RIF in detecting rifampicin resistance is very high (98%) [70,122]. However, emerging data have shown that Xpert MTB/ RIF detects some rifampicin-resistant strains that are identified as susceptible by phenotypic DST. Sequencing these discordant

results usually resolves in favour of Xpert MTB/RIF, and patients missed by phenotypic DST have poor treatment outcomes on first-line treatment [124, 125, 126]. A study by Rigouts and colleagues showed that an epidemiologically-significant proportion of rifampicin-resistant strains (10-13%) in patients who have experienced their first treatment failure and in relapsed patients may be missed by rapid phenotypic DST [127]. The adoption of Xpert MTB/RIF does not eliminate the need for phenotypic DST. Culture remains necessary for treatment monitoring of TB patients, since it is unlikely that any currently available test that uses DNA detection will be suitable for monitoring treatment. In addition, culture-based DST will be required to detect resistance to anti-TB agents other than rifampicin including isoniazid and second-line anti-TB agents, though WHO recommends molecular tests as initial testing also for these drugs, complemented by phenotypic DST if available. Ideally, phenotypic DST for first and second-line drugs should be performed at least for patients diagnosed with rifampicin resistance using molecular methods.

Table 2. Technologies reviewed by the WHO for the diagnosis of active TB and detection of drug resistance.

Test	Assay principle	Use	Sensitivity(%)	Specificity(%)	TAT	References
Imaging techniques						
Chest X-ray	Imaging of the lungs	Active TB disease Screening	87 (using TB abnormality as a threshold)	89 (using TB abnormality as a threshold)	Same day	128
Microscopy						
Conventional sputum smear microscopy	Direct visualization of Mycobacteria using light microscopy	Active TB disease Diagnosis	32–94	50–99	Same day	129
LED fluorescence Smear microscopy	Direct visualization of mycobacteria using fluorescence microscopy	Active TB disease Diagnosis	52–97	94–100	Same day	129
Culture-based techniques						
Liquid culture with DST	Mycobacterial culture on liquid media	• Active TB disease diagnoses • Drug resistance TB detection	• 89 (among smear-positive) • 73 (among smear-negative	>99	10-21 days*	130
Solid culture on L-J media	Mycobacterial culture on L-J media	• Active TB disease diagnoses • Drug resistance TB detection	• 75.8	>98	17–56 days*	57
Microscopic observation of drug susceptibility	Inoculation of specimens to media followed by microscopic examination	• Drug resistance	• 98 for detection of RIF resistance • 91 for detection of INH resistance	99	5–38 days	113, 131
Nitrate reductase assay (NRA)	The ability of *M. tuberculosis* to reduce nitrate on solid culture	• Drug resistance	• 97 for detection of RIF resistance • 97 for detection of INH resistance	99	10–23 days	113, 131

Table 2 (continued). Technologies reviewed by the WHO for the diagnosis of active TB and detection of drug resistance.

Test	Assay principle	Use	Sensitivity (%)	Specificity (%)	TAT	References
Antigen detection techniques						
LAM lateral flow assay	Antigen Detection	Active TB disease diagnosis in HIV-positive individuals	• 44 (all) • 54 (in HIV-positive)	• 92 (all) • 90 (in HIV-positive	Same day	132
Molecular techniques (NAATs)						
Xpert MTB/RIF	NAAT (qPCR)	• Active TB disease diagnosis • Drug resistance (rifampicin)	• 98 (smear-positive & culture positive) • 67 (smear negative & culture-positive) • 95 (RIF resistance)	• 99 • 98(RIF resistance)	Same day	133
First-line LPA (GenoType MTBDRplus and NIPRO)	NAAT (LPA)	• Active TB disease diagnosis • Drug resistance (isoniazid and rifampicin)	• 98 (RIF resistance) • 84 (INH-resistance)	• 99 (RIF resistance) • >99 (INH resistance)	1-2 days	134
Second-line LPA (GenoType MTBDRsl)	NAAT (LPA)	Drug resistance (fluoroquinolones and second-line injectable drugs)	• 86 (fluoroquinolone resistance) • 87 (second-line injectable drugs)	• 98(fluoroquinolone resistance) • 99 (second-line injectable drugs)	1-2 days	135
Loopamp MTBc Assay	NAAT (LAMP)	Active TB disease Diagnosis	70-80	97-98	Same day	136

DST=drug susceptibility testing; LAM=lipoarabinomannan; LAMP=loop-mediated isothermal amplification; LED=light-emitting diode; LPA=line probe assay; NAAT=nucleic acid amplification test; qPCR=quantitative PCR; TAT=turnaround time; TB=tuberculosis. *The TAT indicated is for primary Mycobacterium isolation.

1.4. Treatment of tuberculosis

The primary goals of anti-TB chemotherapy are to kill tubercle bacilli rapidly, prevent the emergence of drug resistance, and eliminate persistent bacilli from the host's tissues to prevent relapse [137]. To accomplish these goals, multiple anti-TB drugs must be taken for a sufficiently long time. The standard treatment regimen for all patients is made up of an intensive phase lasting 2 months and a continuation phase lasting 4 months. During the intensive phase 4 drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) are used to rapidly kill the tubercle bacilli. Infectious patients become less infectious within approximately 10-14 days of starting treatment and symptoms abate [138]. However, the majority of patients with sputum smear-positive TB will become smear-negative within 2 months. In the continuation phase, 2 drugs (isoniazid and rifampicin) are used, over a period of 4 months. The sterilizing effect of these drugs eliminates the remaining bacilli and prevents subsequent relapse [138, 139]. Treatment efficacy and progress are usually monitored with repeat sputum smears. Patients who convert to smear negative and/or improve clinically at the end of the second month are treated with isoniazid and rifampicin in fixed dose combinations given 7 days a week for four months. It is critical that patients have clinical evaluations at least monthly to identify possible adverse effects of anti-TB medications and to assess adherence.

1.4.1. Treatment of EPTB

The basic principles that underlie the treatment of PTB also apply to extrapulmonary forms of the disease. Although relatively few studies have examined treatment of EPTB, increasing evidence suggests that 6 to 9 month regimens (2 months of INH, RIF, PZA, and EMB followed by 4 to 7 months of INH and RIF) are recommended [140, 141]. For treatment of tuberculous meningitis a 9 to 12 month regimen is recommended [140, 141]. Prolongation of therapy also should be considered for patients with TB in any site that is slow to respond. The addition of corticosteroids is recommended for patients with tuberculous pericarditis and tuberculous meningitis. For patients with EPTB the frequency and kinds of evaluations will depend on the site involved. Bacteriological evaluation of the response to treatment in EPTB is often limited by the difficulty in obtaining follow-up specimens. Thus, response often must be judged on the basis of clinical and radiographic findings.

1.4.2. Treatment of MDR- and XDR-TB

Treatment of MDR-TB, which is defined as resistance to both rifampicin and isoniazid, is complex, long, expensive, toxic and ineffective [142]. Patients with MDR-TB are treated with a combination of second-line drugs, usually for 18 months or more. In case of individualized MDR-TB treatment, the choice of drugs should depend on the DST of the strain isolated from the patient or close contacts with MDR-TB, or previous use of the drug, while standardized MDR-TB treatment regimens should build on knowledge about the frequency of its use or documented background drug resistance in the setting. The second-line drugs should include a later-generation fluoroquinolone (such as moxifloxacin, levofloxacin or gatifloxacin), an injectable agent (such as amikacin, kanamycin or capreomycin) and two or more core second-line agents (such as ethionamide, prothionamide, cycloserine, terizidone, clofazimine or linezolid). The recommended duration of treatment is guided by smear and culture conversion which is defined as two consecutive negative smears and culture taken 30 days apart. Attempts to reduce the length of conventional MDR-TB regimens of 18-24 months duration, and to use a combination of drugs which are better tolerated have been ongoing for several years through various studies. Recently, a standardized shorter MDR-TB regimen of 9–12 months is recommended for all patients (excluding pregnant women) with pulmonary MDR/RR-TB not being resistant to second-line drugs. It has shown promising resulted in selected MDR-TB patients [143].

Emergency of so called XDR-TB, which is defined as MDR-TB with additional resistance to any fluoroquinolone and to at least one injectable second-line drug, further complicates the choice of effective therapy. XDR-TB is extremely difficult and expensive to treat and has a very high mortality. Treatment outcomes for patients with XDR-TB are poor; of a cohort of 200 patients with XDR-TB treated in 14 countries, the treatment success rate was only 33%, with high mortality (26%) [144].The two new classes of drugs such as delamanid and bedaquiline are recommended to treat MDR- and XDR-TB under specific conditions in adults [145]. Delamanid and bedaquiline have been proven to be effective in increasing sputum smear and culture conversion, and in improving success rates at the end of treatment, though with some possible side effects [145, 146]. Bedaquiline was approved by the Food and Drug Administration (FDA) in 2012, and the WHO issued guidance on use of the drug in 2013; Delamanid received conditional approval from the European Medicines Agency in 2014 [147]. Delamanid and bedaquiline represent hope for better outcomes but still further clinical trials are required.

1.5. Genotyping of MTBc strains

MTBc strains are characterized by relatively low levels of sequence diversity. Despite this, different MTBc strains can display pronounced phenotypic variation, including differences in their host ranges. The causative agent of human TB, MTBc, comprises seven phylogenetically distinct lineages associated with different geographical regions. Lineages 2 and 4 are the most geographically widespread of all human MTBc lineages, with lineage 4 found on all inhabited continents [148, 149]. Molecular strain typing (genotyping) is important to understand the evolutionary background of clinical isolates and phylogeographical distribution of dominant circulating strains [150]. Different genetic variants of MTBC have been associated with different human populations, potentially reflecting some degree of host–pathogen co-evolution. Significant differences among MTBc lineages have been reported in terms of their virulence, their progression to active disease, and their recognition by the human immune system. There is clear evidence that different strains of MTBc differ in virulence and immunogenicity in experimental infection models. Many other studies have also reported associations between MTBc genotypes and disease phenotypes [151, 152]. Coscolla and Gagneux reviewed nearly one hundred published reports and failed to find a clear association between MTBc lineages and TB disease presentation [153].

Several methods for genotyping of MTBc have been developed over the past years [154]. Spoligotyping is the widely used PCR-based reverse-hybridization blotting technique. It is a fast, robust, and cost effective genotyping technique, but has lower discriminatory power [154]. In the past few years, a new genotyping technique based on PCR amplification of mycobacterial interspersed repetitive units of variable- number tandem repeats (MIRU-VNTR) was introduced [155]. MIRU-VNTR analysis has been shown to have better discriminatory power especially when combined with spoligotyping. Ethiopia harbours a vast human genetic diversity and has a high percentage of EPTB, dominated by cervical lymphadenitis. In Ethiopia, only limited data is available on the association of particular MTBc strains and their ability to cause EPTB.

1.6. Justification of the studies

The global priorities for TB care and control are to improve case-detection and to detect cases earlier, including cases of smear-negative and EPTB disease, and to enhance the capacity to diagnose MDR-TB [156]. Despite increases in notifications of TB and MDR/RR-TB, big detection and treatment gaps remain the latest. WHO report estimated that approximately 4 million people with TB are either not diagnosed, diagnosed too late, or are not known to the TB control program. Moreover, less than 50% of estimated MDR/RR-TB cases were started on treatment in 2015 [23]. Main reasons for these gaps are inadequate diagnostic capacity and an over reliance on chest radiography and/or sputum smear microscopy as diagnostic tools. Patients with sputum smear-negative and/or extra-pulmonary disease and drug-resistant TB patients are particularly affected by the failure of microscopy as primary diagnostic tool.

Despite a reduction in Ethiopia's annual TB mortality rate, TB still causes more than 30,000 fatalities annually- more than 80 TB deaths every day [35]. Smear-negative PTB and EPTB comprise up to 70% of the cases registered annually in Ethiopia compared to 50% worldwide [30]. In Ethiopia- as in most settings - TB control relies on passive case finding among individuals self-presenting to health care facilities, followed by either diagnosis based on clinical symptoms, chest-X-ray or laboratory diagnosis using sputum smear microscopy. Although smear-microscopy is a relatively quick and easy method, it misses many cases. In addition, TB is more difficult to diagnose in individuals with smear-negative and EPTB [157].

Table 3: Impact of Xpert MTB/RIF in case detection (initial results) in Ethiopia.

	Before Xpert implementation (Jul-Dec, 2013)	After Xpert implementation (Jul-Dec, 2014)	% change
Total DR- TB detected	254	311	22% increment
RR-TB detected	65	268	4-fold increase
Xpert MTB/RIF contribution for DR-TB cases detected	25.6%	86.2%	Above 3-fold increase
TB cases detected	58,802	63,168	7.5% increment

Xpert MTB/RIF contribution to TB case detection from July 2013-July 2014 was 2%.

Key: DR=drug resistant TB, RR=rifampicin resistant TB.

Source: *Presentation by Endale Mengesha at GLI/GDI meeting, Geneva, April 2015.*

Detecting more cases, detecting them early, and rapidly identifying drug resistance are essential for improving individual patient health and avoiding transmission in the community. Although the latest generation liquid culture diagnostics and molecular li ne probe assays are recommended by WHO for rapid detection of TB and MDR-TB, they have not yet solved the diagnostic dilemma in most resource-limited settings, largely due to the need for expensive laboratory infrastructure, extensive biosafety precautions, and specialized staff [158].The Xpert® MTB/RIF assay represents a paradigm shift in the diagnosis of TB and drug-resistant TB by simultaneously detecting *M. tuberculosis* and rifampicin resistance-conferring mutations in less than two hours directly from clinical samples [159, 160].

Improving TB detection rates and further reducing the burden of disease in Ethiopia will require optimization of the current laboratory techniques as well as the systematic introduction of new diagnostic technologies or methods with improved sensitivities and specificities. For more effective control of TB in Ethiopia, the diagnosis of TB in difficult to diagnose groups such as EPTB and smear-negative TB should be improved. The diagnosis of EPTB remains more challenging since clinical specimens from deep-seated organs may be difficult to obtain or require specialized skill and equipment that are not available in resource-poor settings. Investigating simple and efficient way to enhance the sensitivity of the conventional and new methods (such as conventional smear- microscopy, light emitting-diode based fluorescent microscopy and Xpert MTB/RIF) in paucibaccilary samples should be taken in account.

Following the WHO recommendation, the Ethiopian Federal Ministry of Health has endorsed the Xpert MTB/RIF test in 2014 and developed an implementation guideline to facilitate the introduction of this technology into the existing TB diagnostic system throughout the country. In developing countries like Ethiopia it may not be cost effective to test every TB suspected individual by Xpert MTB/RIF. Maximum benefit from the Xpert MTB/RIF assay is obtained by targeted testing of individuals considered at risk of TB (drug resistant TB) and analysing the specimen that yield higher sensitivity. Thus determining the best possible indications to test for TB or MDR-TB with Xpert MTB/RIF so as to make the most efficient use of Xpert MTB/RIF testing is an important concern.

Moreover, the cause for the very high rate of tuberculous lymphadenitis in Ethiopia has not been systematically investigated. We tested the hypothesis whether the high rate of TB lymphadenitis in Ethiopia is linked to specific genotypes of MTBc, such as *M. bovis* or lineage 7 isolates.

1.7. Goals and objectives

1.7.1. Goal

- To improve the diagnosis and clinical management of smear-negative pulmonary and EPTB by optimizing the use of GeneXpert MTB/RIF and alternative methods in Ethiopia

1.7.2. Objectives

1. To evaluate the accuracy of Xpert MTB/RIF and the added value of bleach concentration on the performance of Xpert MTB/RIF for the diagnosis of smear-negative PTB (**Paper I**)
2. To evaluate whether concentration of lymph node aspirate improves the sensitivity of acid fast smear microscopy for diagnosing tuberculous lymphadenitis (**Paper II**)
3. To determine the diagnostic performance of light-emitting diode (LED)-fluorescent microscopy on routinely collected fine needle aspirates (**Paper III**)
4. To determine the performance of Xpert MTB/RIF for the diagnosis of tuberculous lymphadenitis from concentrated lymph node aspirate (**Paper IV**)
5. To determine the clinical utility of Xpert MTB/RIF for the diagnosis of EPTB in Ethiopia (**Paper V**)
6. To investigate the genetic diversity of MTBc isolates causing tuberculous lymphadenitis in Southwest Ethiopia (**Paper VI**)

Chapter two: General Methods and Materials

2.1. Study area

Ethiopia is located in the North Eastern part of Africa, also known as the Horn of Africa. Ethiopia covers an area of 1.1 million square km and has a population of 99 million people. Ethiopia is a predominantly agricultural country and more than 80% of the population lives in rural areas. Administratively the country is divided into nine Regional States and two City Administrative Councils (Addis Ababa and Dire Dawa). Each Regional State is further divided into Zones, then Woredas and Kebeles (the lowest administrative unit).

The study area is located in Jimma Zone, Oromia Regional State in Southwest direction of Ethiopia. Oromia is the largest and most populous region in Ethiopia. It covers 359,619 square km (32% of the country) and has a population of about 34.88 million. The Oromia region is administratively subdivided into 20 zones, 12 towns, 342 Woredas and 7021 Kebeles. According to a more recent report, there are 66 hospitals, 1354 health centers, 6622 health posts, 4149 private clinics and 2 regional laboratories in Oromia Regional State (**Table 4**). The potential health service coverage is 70.5% and the health service utilization is 27% [161].

Jimma zone is located in Oromia Regional State. This zone has a total population of about 3.3 million, with a population density of 159.69, and covers an area of 15,568.58 square km. Jimma zone has 18 districts, two towns (Jimma and Agaro) and 530 Kebeles. Jimma town is the largest city in Southwest Ethiopia and is a special zone of the Oromia Regional State. Jimma town is located 357 km away from Addis Ababa (the capital city of Ethiopia). The town is located at an altitude of about 1780 meter above sea level and has a latitude and longitude of 7°40'N 36°50'E. Jimma town has a warm and humid climate with a mean annual maximum temperature of 33°C and a mean annual minimum temperature of 10°C. It lies in the climatic zone locally known as 'Woyna Daga' which is considered ideal for agriculture as well as human settlement [162].

Jimma town has two governmental hospitals and four health centres that provide health care services for people living in Jimma and the surrounding areas. Jimma University Specialized Hospital (JUSH), located in Jimma town, is one of the oldest public hospitals in the country. It was established in 1938 during the Italian occupation for the service of their soldiers. Later

JUSH was owned by Jimma University and since then, the university has made relentless efforts, through extensive renovation and expansion work to make the hospital conducive for service, teaching and research. Currently, it's the only teaching and referral hospital in the Southwestern part of the country. It provides specialized health services through its nine medical and other clinical and diagnostic departments for approximately 15,000 inpatient, 160,000 outpatient attendants, 11,000 emergency cases and 4500 deliveries per year from a catchment population of about 15 million people. It has a bed capacity of 600 and a total of more than 1448 staff, including both supportive and professional staff [163].

Laboratory investigations for most of the studies in this thesis were carried out at Mycobacteriology Research Center of Jimma University (JU-MRC), Ethiopia. The MRC was established as part of a collaborative research project between Jimma University and a consortium of Flemish Universities from Belgium in November 2010. The center activities are mainly focused on basic research, training and service in the field of Mycobacteriology. The research component of the center is mainly on basic and applied research which ranges from optimization of detection methods to molecular level research. The center is also open for training on Mycobacteriology techniques up on request by academic departments. It is also involved in the provision of service to the patients as a part of a national Mycobacteriology laboratory network. Conventional bright field microscopy, fluorescent microscopy, culture to detect *M. tuberculosis* using solid media (L-J and Middlebrooke media) and liquid media (BACTEC MGIT 960 TB detection system); DST on solid media, liquid media and line probe assay (GenoTypeMTBDRplus assay), and Xpert MTB/RIF are the currently available technologies at the center [164].

Figure 4: Map showing the study area

Table 4: The capacity of health facilities providing diagnostics and treatment of TB and MDR-TB in Oromia Region, Ethiopia.

Types of HFs	Total HFs	AFB microscopy and treatment services	TB treatment service only	Fluorescence microscopy centers	MDR-TB TICs	Current MDR-TB TFCs
Hospitals (Gov't)	55	51	0	51	17	34
Health center (Gov't)	1,354	968	331	150	0	196
Total Public HFs	1409	1019	331	201	17	230
Private Hospital	19	19	0	0	0	0
Private HFs (high & medium)	523	126	0	0	0	0
Lower clinic (primary)	2907	0	0	0	0	0
Total Private HFs	3449	145	0	0	0	0
All Type	4,858	1164	331	201	17	230

HFs= health facilities, TICs=treatment initiating centers, TFCs=treatment follow-up centers

2.1.1. TB situation in the study area

In Oromia region, TB is a priority among public health concerns and Oromia region alone reported more than 37% of the national TB cases in 2016. In Oromia regional sate, a total of 72,203 TB cases of all forms were expected for 2016. However, only 48,039 (66.5%) TB cases were detected and placed on anti-TB treatment. The overall regional case notification rate for the year 2016 was 138 per 100,000 populations. Bacteriologically confirmed TB type, accounted for 37.8% (17885) among all forms of TB. For diagnosis of TB, 1078 health facilities (HFs) (952 public and 126 private) have AFB smear microscopy, 37 HFs have Xpert MTB/RIF test and 2 laboratories are equipped with culture facilities. TB treatment is provided in all hospitals and health centers (DOTS coverage was 100% in hospitals and health centers, and 45% in health posts). Cure and treatment success rates among bacteriologically confirmed TB cases were 90% and 95% respectively. Currently, there are 17 treatment initiation centers (TICs) and 34 treatment follow-up centers (TFCs) for MDR-TB. In the region, MDR/RR-TB case detection rate performance was very low (7% only). Only 66 MDR-RR/TB cases were identified and enrolled during the 6 months period (July 2016- December 2016). Among 44,444 TB cases tested for HIV, 2116 were HIV positive and TB/HIV co-infection rate was 4.8%. Among confirmed MDR/RR-TB cases placed on second line anti-TB treatment, 16 were found to be HIV Positive [165].

In Jimma zone there are 92 HFs (4 hospitals and 88 health centers) that are equipped with AFB smear microscopy and TB treatment services. The zone has only one center (i.e. JU-MRC) for phenotypic DST and ten MDR-TB treatment and follow-up centers. According to a recent Challenge TB report (a non-governmental organization), a total of 237,445 OPD visitors were screened for TB and 7180 (3%) presumptive TB cases were identified in Jimma zone in 2016. A total of 3629 TB cases (all forms) were notified for the year 2016 in Jimma zone, of which 1341 were smear positive pulmonary TB cases. Among treated TB cases, cure rate (CR) of 94.8% and treatment success rate (TSR) of 96.6% were reported in 2016 (**Table 5**). Among the total of 66 presumptive MDR-TB cases who had been identified, only 24 (36%) had full DST results. In the same period, 167 new people living with HIV were enrolled in Jimma zone and 111 (66.5%) were started on IPT [166].

Table 5: Latest estimates of tuberculosis situation in Oromia regional state, 2016

Total population	34,880,772
Number of health facilities	4,858
Number of AFB smear microscopy and TB treatment services sites	1164
Number of Xpert MTB/RIF testing sites	37
TB culture performing laboratories	2
Total number of TB cases expected for the year 2016	72,203
Total TB cases detected and placed on anti-TB treatment for the year 2016	48,039
Bacteriologically confirmed pulmonary TB (BCPTB)	17,885 (37%)
Clinically diagnosed pulmonary TB (CDPTB)	14,899 (31%)
Extrapulmonary TB (EPTB)	14,077 (29.5%)
Relapse	896 (1.8%)
Total TB case notification rate per 100,000 population in 2016	138
Percent of TB case detection through community TB care	49%
Case detection rate (CDR)	66.5%
TSR among new bacteriologically confirmed pulmonary TB cases	95%
Percent of TB patients tested for HIV	93%
TB/HIV co-infection rate	4.8%
Percent of Newly enrolled HIV patients put on IPT	42.4%
Percent of HIV-positive TB patients on Ant-Retroviral Therapy (ART)	86%
Number (%) of TB cases (all forms) diagnosed through private health facilities	5%
Number of presumptive DR TB identified	4065
Number of confirmed RR TB only cases	134
Number of confirmed MDR TB cases	99
Number confirmed MDR-TB or RR/TB put on second line treatment	196
MDR-TB cohort cases enrolled on second-line anti-TB treatment	114

Source: Challenge TB Regional Review Meeting in Oromia Region, October 24-28, 2016

Table 6: Latest estimates of tuberculosis situation in Jimma zone, 2016

Total population	3,350,076
Number of health facilities	124
Total number of OPD visitors	249,238
Number and percent of OPD visitors screened for TB	237,445
Number and percent of presumptive TB cases identified	7180 (3%)
All forms of TB cases notified	3629
Bacteriologically confirmed pulmonary TB (BCPTB)	1676
Clinically diagnosed pulmonary TB (CDPTB)	837
Extrapulmonary TB (EPTB)	1072
Relapse	44
Case detection rate (CDR)	52.3%
Community contribution in case detection (%)	24%
Private Health facilities contribution (%) in TB cases diagnosis & treatment	2.2%
Case notification rate (CNR) all forms of TB	88%
CR for Bacteriologically confirmed PTB	94.8%
TSR for Bacteriologically confirmed PTB	96.6%
Number of Presumptive MDR-TB identified in 6 months (Jul-Dec, 2016)	66
Number of Presumptive MDR-TB with DST result (Jul-Dec, 2016)	24 (36%)
TB/HIV co-infection rate	1.4%
TB/HIV co-infected put on ART	100%

Source: Challenge TB Regional Review Meeting in Oromia Region, October 24-28, 2016

2.2. Summary of study design, study subjects and laboratory investigations

Patients visiting Jimma University Specialized Hospital (JUSH) from the different areas of Southwest Ethiopia for routine clinical care were our broader study population. Upon arrival at JUSH, all patients were clinically examined and those suspected of pulmonary or extrapulmonary TB were enrolled in the current studies. In the first study (**Chapter three**), a total of 326 consecutive pulmonary TB suspected patients were screened for TB with conventional smear microscopy. Of these, those 185 patients who had three times sputum-smear negative results on AFB microscopy were included. Two sputum samples (one spot and

one morning) were collected per patients. The first sputum was analyzed for direct Xpert MTB/RIF and culture (the gold standard). Half of the second sputum was used for direct Xpert MTB/RIF and the remaining was treated with bleach and concentrated by simple centrifugation. The concentrated sediment was tested by Xpert MTB/RIF.

In the second study (**Chapter four**), lymph node specimen was collected from 200 patients clinically suspected of having tuberculous lymphadenitis. The first few drops of the aspirate were used for cytomorphological study and direct smear-microscopy. The remaining aspirate was treated with N-acetyl-L-cysteine (NALC) and concentrated by centrifugation at 3000g for 15 minutes. The sediment was used for smear-microscopy (ZN) and culture (L-J medium). The accuracy of AFB smear microscopy on concentrated lymph node aspirate was calculated using culture (L-J) as gold standard.

In **Chapter five**, we assessed the diagnostic performance of light-emitting diode (LED) fluorescent microscopy on routinely collected fine-needle aspirates from 144 tuberculous lymphadenitis presumptive cases. Three direct FNA smears per patient were performed and subjected for cytology, conventional Ziehl–Neelsen microscopy and LED-fluorescence microscopy. The remaining aspirate was processed for culture on L-J (gold standard).

In our fourth study (**Chapter six**), we evaluated the performance of Xpert MTB/RIF for the diagnosis of tuberculous lymphadenitis on concentrated fine needle aspirate (FNA). FNA sample was collected from 143 patients suspected of having lymph node TB. The FNA samples were concentrated in the same manner as it was done in Chapter four and used for culture and Xpert MTB/RIF. The diagnostic accuracy of Xpert MTB/RIF was calculated using composite bacteriological methods (culture and/or smear microscopy) as reference standard.

Having promising results of Xpert MTB/RIF on processed FNA samples, we became interested in investigating the overall performance Xpert MTB/RIF for diagnosing EPTB using different types of extra-pulmonary specimens (**Chapter seven**). Extra-pulmonary specimens were collected from 572 patients with clinically presumed EPTB. These comprised 279 lymph node specimens, 45 cerebrospinal fluid (CSF), 160 pleural, 80 peritoneal and 9 pericardial specimens. All specimens were processed for the following tests: fluorescence staining for AFB, mycobacterial culture (BACTEC 960 MGIT) and Xpert MTB/RIF at Jimma University-Mycobacteriology Research Center. The diagnostic accuracy of Xpert MTB/RIF was calculated

compared to culture and a composite reference standard (CRS). The CRS for this study was composed of liquid culture (MGIT 960), smear microscopy, cytology, clinical and radiological findings and clinical response. For Xpert MTB/RIF-rifampicin resistance cases, further drug susceptibility testing by the GenoTypeMTBDR*plus* line probe assay was performed on DNA extracts from a positive culture.

We also studied the genetic diversity of MTBc isolates causing tuberculous lymphadenitis in Southwest Ethiopia (**Chapter eight**). FNA was collected from the swollen lymph nodes of 436 consecutive patients presumptive of having TB lymphadenitis and cultured in MGIT960 tubes. DNA was extracted from 310 culture-positive MTBc isolates at Jimma University-Mycobacteriology Research Center, Ethiopia. DNA extracts were transported to the Institute Tropical Medicine (ITM), Antwerp, Belgium for spoligotyping (n=305) and qPCR-based single nucleotide polymorphism detection (n=38). Isolates of selected spoligotypes (n=167) were further genotyped by 15-loci mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) at Genoscreen, Lille, France. Isolates were classified into main phylogenetic lineages and families by using the reference strain collections and identification tools available at *MIRU-VNTRplus* data base. Table 7 shows the summary of the study design, study population and methodology of studies considered in this thesis.

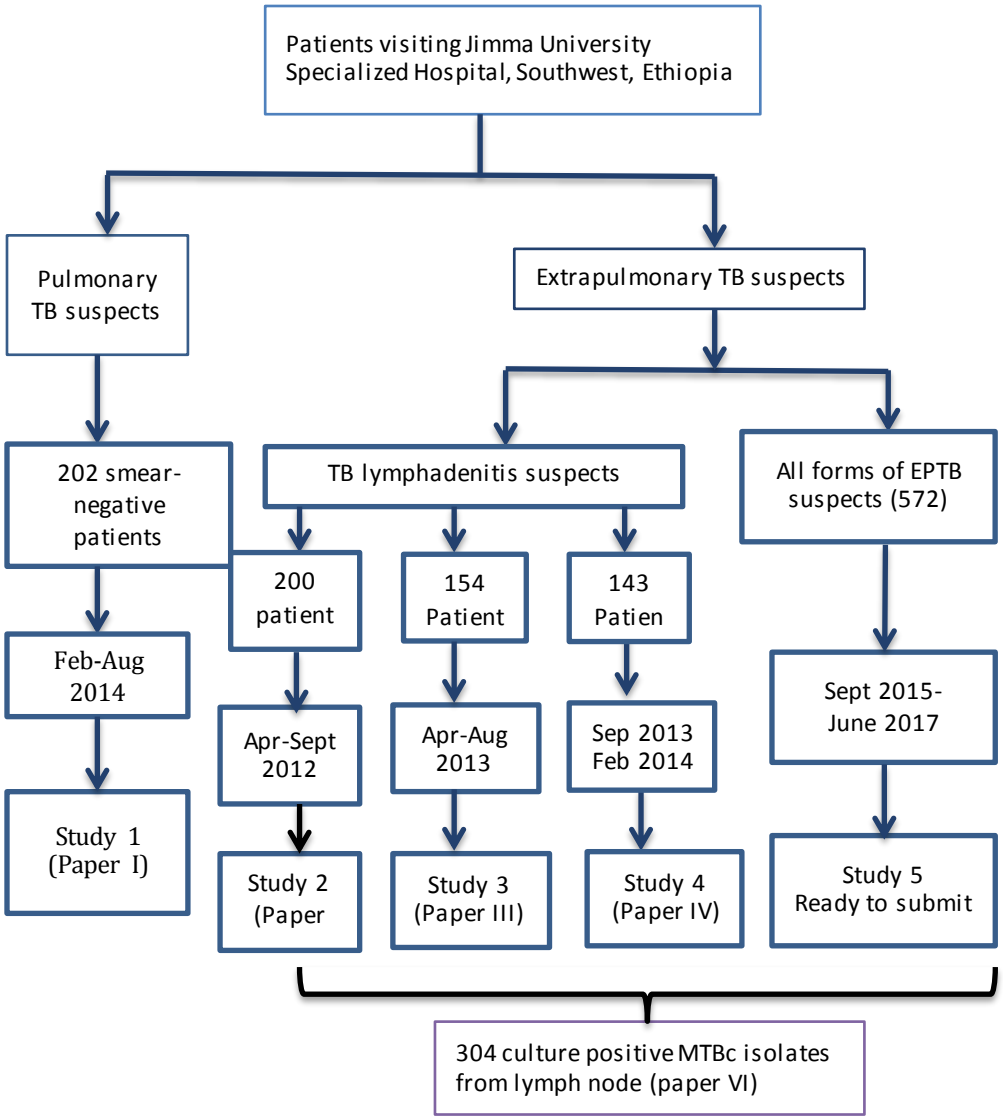

Figure 5: Flowchart describing the overall studies included in this thesis

Table – 7: Summary of the studies conducted: the study population and sample size, the diagnostic tool evaluated and the gold standard method.

Papers	Paper title	Study population and sample size	Diagnostic tool evaluated	Gold standard	Publication status
Paper I	Increased detection of smear-negative pulmonary tuberculosis by Xpert MTB/RIF® assay after bleach-concentration	Smear-negative pulmonary TB suspects (n=185)	Xpert MTB/RIF	L-J and/or MGIT culture	Published in *International Journal of Mycobacteriology*
Paper II	Concentration of lymph node aspirate improves the sensitivity of acid fast smear microscopy for the diagnosis of tuberculous lymphadenitis in Jimma, Southwest Ethiopia	Clinically suspected cases of tuberculous lymphadenitis (n=187)	Conventional smear-microscopy	L-J culture	Published in *PLOS/ONE*
Paper III	Diagnostic performance of fluorescent light emitting diode microscopy for tuberculous lymphadenitis in a high burden setting	Clinically suspected cases of tuberculous lymphadenitis (n=144)	LED-Fluorescence Microscopy	L-J culture	Published in *Journal of Tropical Medicine & International Health*
Paper IV	GeneXpert MTB/RIF assay for the diagnosis of tuberculous lymphadenitis on concentrated fine needle aspirates in high TB burden setting	Clinically suspected cases of tuberculous lymphadenitis (n=143)	Xpert MTB/RIF	L-J and/or AFB microscopy	Published in *PLOS/ONE*
Paper V	Clinical utility of Xpert MTB/RIF for diagnosis of extrapulmonary tuberculosis in Ethiopia	Clinically suspected of having EPTB (n=572)	Xpert MTB/RIF	Liquid culture or CRS**	Manuscript draft ready to submit
Paper VI	The predominance of Ethiopian specific *M. tuberculosis* families and minimal contribution of *M. bovis* in tuberculous lymphadenitis patients in Southwest Ethiopia	Culture confirmed MTBc isolates (n=304)	NA	NA	Published in *Infection, Genetics and Evolution (MEEGID Journal)*

L-J: Lowenstein–Jensen, MGIT: Mycobacteria Growth Indicator Tube, LED: light emitting diode, NA: note applicable, **CRS (composite reference standard) composed of liquid culture (MGIT960), microscopy, cytological and radiological findings and treatment response.

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Chapter three: Paper I

3. Increased detection of smear-negative pulmonary tuberculosis by GeneXpert MTB/RIF® assay after bleach-concentration

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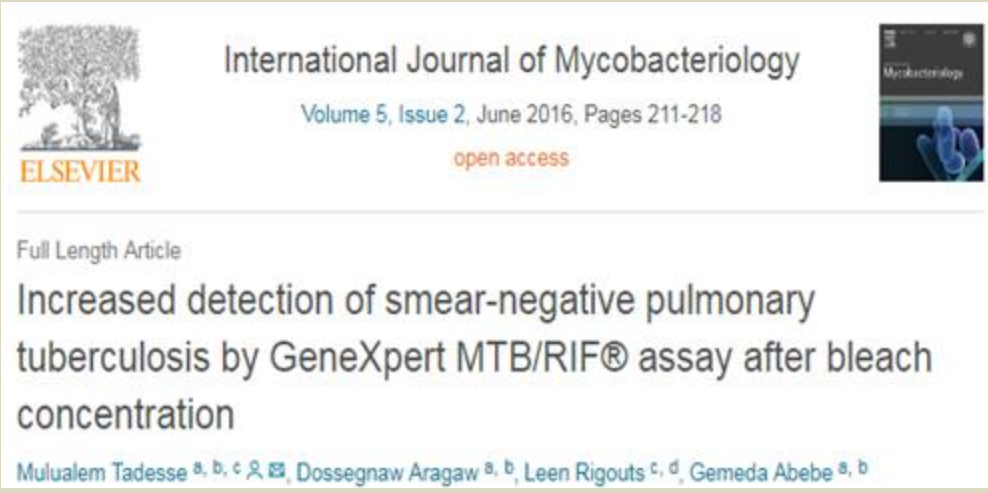

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Performed the experiments and analyzed the data: DA, MT

Coordinated the microbiological work: GA, LR

Wrote the initial draft: MT, DA

Critically reviewed the manuscript: GA, LR

3.1. Abstract

Background: The GeneXpert MTB/RIF® assay (Xpert MTB/RIF) was endorsed as the initial diagnostic tool in people suspected of HIV-associated or drug resistant tuberculosis (TB). However, information regarding the performance of Xpert for diagnosing smear-negative TB in high burden settings remains limited. We evaluated the diagnostic accuracy of Xpert MTB/RIF and the impact of bleach-concentration on the performance of Xpert MTB/RIF using smear-negative sputum sample from HIV-negative patients.

Methods: One spot and one morning smear-negative sputum samples per patient were examined using Xpert MTB/RIF and culture at Mycobacteriology Research Center of Jimma University, Ethiopia. The sputum culture on both Löwenstein-Jensen (LJ) and/or mycobacteria growth indicator tube (MGIT) was the gold-standard.

Results: Of 185 smear-negative presumptive pulmonary TB cases, 19 (10.3%) had culture-proven TB. The sensitivity of Xpert MTB/RIF on spot and morning sputum was similar (63.2%). Testing two specimens per patient insignificantly increased the sensitivity of Xpert MTB/RIF. Bleach-concentration and pelleting improved sensitivity of Xpert MTB/RIF over unprocessed sputum in paired samples (73.8% vs 63.2%) without affecting the specificity (95%). Bleach-concentration and pelleting allowed an additional 7 cases of TB (missed on the first and second direct Xpert MTB/RIF) to be detected, 5 of which were from culture-negative cases.

Conclusions: Testing of single sputum sample by Xpert MTB/RIF can reach reasonable sensitivity and result would be available on the same day, avoiding loss of patients and treatment delay. The sensitivity of Xpert MTB/RIF was improved after bleach-concentration and pelleting, though its added value needs further study on a larger scale.

Key words: Smear-negative tuberculosis, Xpert MTB/RIF, diagnostic accuracy, bleach-concentration

3.2. Introduction

Tuberculosis (TB) remains a major public health problem, accounting for 9 million incident cases and 1.5 million deaths worldwide [1]. Ethiopia ranks 10th among the 22 countries with high TB burden [2, 3]. Recently, the number of registered smear-negative TB cases transcended the smear-positive cases. This is a peculiar situation seen in Ethiopia for over a decade. In the national TB prevalence survey in 2010/11 smear-negative cases accounted for 57% of culture-positive cases [4]. Diagnosis of smear-negative pulmonary TB remains a challenge [3, 5, 6]. This is because of the lack of rapid and accurate diagnostic modalities that can be applied in resource-limited settings [3, 7].

The GeneXpert MTB/RIF® assay (Cepheid, USA; hereafter referred to as 'Xpert') detects the presence of *Mycobacterium tuberculosis* complex (MTBC) and its resistance to rifampicin in a single reaction [8, 9]. Xpert is an integrated fully automated specimen processing and nucleic-acid-amplification test [8, 10], strongly recommended by WHO for diagnosis of HIV-associated TB and multidrug-resistant TB [11]. In clinical evaluation studies, the sensitivity of Xpert in patients with smear-negative pulmonary TB was reported to be moderate, 55–86%, as compared to 99–100% in patients with smear-positive TB [12-14]. This urges for a simple and efficient way to enhance the sensitivity of Xpert for detection of *M. tuberculosis* in smear-negative patient.

House hold sodium hypochlorite (NaOCl) or bleach has been used for over a century to increase the yield of microscopic detection. Bleach at 5% concentration can digest the sputum products and inactivate the mycobacteria without altering their structures. This provides a greater safety for laboratory use. Further centrifugation or sedimentation concentrates the acid fast bacilli (AFB) in the mixture, increasing the rate of positivity [15]. In the current study, we primarily evaluated the diagnostic accuracy of direct Xpert test in smear-and HIV-negative patients. Secondly, we determined whether sputum processing using bleach combined with simple centrifugation can increase the sensitivity of Xpert in resource poor settings.

3.3. Materials and Methods

3.3.1. Study participants

A cross-sectional prospective study was carried out at Jimma University Specialized Hospital, a public tertiary care hospital, in Southwest Ethiopia. Adult consecutive patients with

presumptive pulmonary TB presenting at the health care facility were screened between February and August, 2014. Inclusion criteria were based on the WHO case definition for presumptive pulmonary TB [16] and included patients having a persistent cough for at least 2 weeks with or without one of the following: night sweats, unintentional weight loss, fever, chest pain, shortness of breath, loss of appetite, and contact with a TB patient.

Consenting patients were enrolled only if they had three times sputum-negative result on smear microscopy, were HIV-negative, and could provide detailed clinical history along with an adequate amount of sputum specimen. Demographic and clinical characteristics were collected through interview by using a pre-tested questionnaire. This study was approved by institutional review boards of Jimma University, Ethiopia (Ref. No: RPGC/510/2014). All participants gave written consent for use of routine clinical data for research purposes.

3.3.2. Sputum sample processing

Portion of the collected sputum specimen was used immediately for direct smear microscopy and the remaining portion was stored at 4^0C in a refrigerator for culture and Xpert. HIV test results were collected from the medical records after obtaining written consent from the clients. One spot and one morning sputum sample collected from each presumptive TB case were brought to room temperature and liquefied by N-acetyl L-cysteine (NALC) powder. Then each sputum sample was divided in to two aliquots, which were carefully labeled and assayed independently in a blinded manner by a second person. The first aliquot of spot sputum was assayed for direct Xpert and the other aliquot for culture (MGIT 960 & LJ medium). Likewise, the first aliquot of morning sputum was tested for direct Xpert and the second for bleach-concentrated Xpert.

3.3.3. Direct Xpert

Xpert was performed as previously described [8]. Sample reagent was added to sputum specimen in a 2:1 ratio in a sterile falcon tube. The solution was vortexed and left to settle for 15 minutes, with vortexing halfway through. The supplied sterile transfer pipette was used to draw the liquefied sample up to the marked line (corresponding to 2ml) and transferred to a cartridge. The Xpert cartridge was then loaded onto the Xpert machine (Cepheid, Dx System Version 4.0c). Results were reported as positive or negative for MTBC. Rifampicin resistance results were reported as susceptible, resistant or indeterminate.

3.3.4. Sputum culture and identification

Mycobacterial culture was done in MGIT 960 and on LJ medium. Sputa were decontaminated by the standard *N*-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method with a final NaOH concentration of 1% [17]. An equal volume of standard NALC/NaOH solution was added to the specimen and incubated for 15 minutes at room temperature. After centrifugation for 15 minutes at 3000g, the sediment was re-suspended in 1ml of sterile phosphate buffered saline (PBS; pH 6.8). The resulting pellet was used to inoculate a MGIT 960 and LJ tube. The *M. tuberculosis* H_{37}Rv reference strain (ATCC 27294) was processed with each run as a positive control. All positive cultures were confirmed for MTBC by SD BIO LINE MPT64 TB Ag test (Standard Diagnostics, Yongin, South Korea).

3.3.5. Bleach-concentrated sputum for Xpert

Xpert from bleach-treated and concentrated sputum was carried out according to the optimized protocol in the validation experiment of this study (described hereunder). Briefly, the second part of the morning specimen was transferred to a conical centrifuge tube (15ml; SARSTEDT) and treated with an equal volume of 5% bleach (Chora Gas and chemical products factory, Addis Ababa, Ethiopia). After mixing, the tube was left for 15 minutes at room temperature with shaking for 30 seconds every five minutes. Then PBS was added up to 15ml and centrifuged at 3000g for 15 minutes using a simple centrifuge (a low cost table top centrifuge that is available in peripheral laboratories of Ethiopia). After centrifugation the supernatant was decanted carefully and the sediment was re-suspended with 1ml of sterile PBS (pH=6.8). Sample reagent was added in a 3:1 ratio and shaken vigorously for 15 seconds. Subsequently, the procedure described for direct Xpert was followed [8].

3.3.6. Statistical analysis

Data were analysed by using SPSS version 20.0. Sensitivity, specificity, positive predictive value, and negative predictive value of Xpert and their respective 95% confidence intervals (CI) were calculated using culture as reference standard. A positive culture result was defined as growth on LJ and/or in MGIT identified as *M. tuberculosis.* We excluded data of patients with contaminated sputum culture results and indeterminate Xpert results from the diagnostic accuracy analysis. The study reporting conforms to the STARD guidelines for diagnostic accuracy reporting (www.stard-statement.org/).

3.3.7. Validation experiments

3.3.7.1. Effect of bleach-sputum processing on Xpert

The protocol was based on pre-treatment of sputum sample with 5% bleach (Chora Gas and chemical products factory, Addis Ababa, Ethiopia). It is known that house hold bleach (sodium hypochlorite) can digest and liquefy mucus and debris in sputum [15] . This makes the TB bacilli to be released, which can be further concentrated by centrifugation. Two experiments were conducted to check whether pre-treatment of sputum with bleach could have an interfering effect on Xpert result.

In the first experiment, a total of ten AFB-negative sputum samples (n=10) spiked with the *M. tuberculosis* $H_{37}Rv$ reference strain were included. Briefly, $H_{37}Rv$ was cultured on LJ medium. Using a sterile wire loop, 3–5 well-isolated colonies were emulsified in 3ml of sterile physiological saline (0.9%w/v). The bacterial suspension's turbidity was matched with that of McFarland No 0.5 (approximately $1x10^8$ CFU/mL). This suspension was spiked into 2ml of liquefied (kept overnight at room temperature) smear negative sputum from a healthy volunteer. Each spiked sample was divided in two groups; one received the treatment with 5% bleach followed by centrifugation (3000g for 15minutes) and the other did not receive any treatment with bleach, nor centrifugation. Bleach-treated and untreated samples were analysed by Xpert (as per manufacturer's instruction). Positive results were placed in one of four categories; very low, low, medium, or high based on the quantitative cycle threshold (*Ct*) value of probe A.

The experiment was expanded by testing known smear-positive sputum specimens (n=9). The sputum samples were kept at room temperature for 24 hours to allow liquefaction. This enabled us to split the sample into two equal parts (approximately 1ml each). As in the case of spiked sputum, the first part was treated with 5% bleach and the other left untreated. Bleach-treated and -untreated samples were tested by Xpert.

3.3.8. Validation results

A total of 19 paired (bleach-treated and-untreated) samples were analyzed by Xpert. The test result showed that all bleach-untreated samples (10 $H_{37}Rv$ spiked smear-negative and 9 smear-positive sputa) and 18 bleach-treated samples (9 $H_{37}Rv$ spiked smear-negative and 9 smear-positive sputa) were Xpert-positive. Result for one of bleach-treated spiked sputum was

uninterpretable (error). The mean Ct value was 13.77 for bleach-untreated and 14.90 for bleach-treated specimens. Bleach-treatment and centrifugation did not seem to affect Ct-values (T-test values of 0.800 and 0.460 for spiked and naturally infected sputa) (**Table 1**). We concluded that 5% bleach did not have an interfering effect on Xpert test result, though more studies are warranted to further validate the protocol. In some of our samples the Ct value was increased after bleach treatment and centrifugation, which could be due to the fact that some mycobacterial cells have lower buoyant density and centrifugation at 3000g for 15 min is unlikely to result in effective recovery these cells.

Table 1. Pre-study Xpert validation results using paired artificially spiked smear-negative and naturally smear-positive sputum samples with or without pre-test bleach treatment.

Code	Sputum appearance	Bleach-untreated		Bleach-treated		Paired T-test
		Result	C_t value	Result	C_t value	*P*-value
H37Rv spiked sputum						
3830	Purulent	Positive	13.4	Positive	16.6	0.080
3854	Bloody	Positive	14.8	Positive	14.7	
3830	Purulent	Positive	13.7	Error	-	
3864	Muco purulent	Positive	14.9	Positive	15.6	
3852	Purulent	Positive	12.9	Positive	12.5	
3830	Purulent	Positive	15.6	Positive	17.4	
3854	Purulent	Positive	15.6	Positive	16.3	
3839	Muco purulent	Positive	19.7	Positive	19.1	
3846	Muco purulent	Positive	9.7	Positive	10.9	
3846	Highly mucoid	Positive	10.4	Positive	16.5	
Smear positive sputum						
3558	Muco Purulent	Positive	15.8	Positive	20.5	0.460
3560	Purulent	Positive	16.2	Positive	14.3	
3577	Muco Purulent	Positive	10.6	Positive	10.9	
3608	Muco Purulent	Positive	13.5	Positive	12.7	
3612	Purulent	Positive	12.7	Positive	16.6	
3669	Purulent	Positive	10.0	Positive	9.0	
3778	Muco Purulent	Positive	12.6	Positive	15.2	
3788	Muco Purulent	Positive	13.9	Positive	18.4	
3836	Purulent	Positive	15.7	Positive	11.0	

Ct= Cycle threshold

3.4. Results

3.4.1. Characteristics of study participants

During the study period, a total 326 consecutive adult patients with presumptive pulmonary TB were screened. One hundred twenty four patients were excluded from the study (56 were HIV-positive/unknown, 30 were smear positive, 19 provided a sample with inadequate volume, 13 did not provide three sputa, and 6 had missing AFB-smear results). Of the remaining 202 presumptive TB patients who had smear-negative sputum, 17 patients were excluded (13 culture contaminated & 4 Xpert MTB/RIF indeterminate results), leaving 185 patients for the final analysis. Overall study flow diagram is shown in **Figure 1.** Majority, 61.6% (114/185), of study participants were males. The median age of patients was 38 years (inter-quartile range (IQR) 23–55). The study participant characteristics are shown in **Table 2.**

Table 2. Demographic and clinical characteristics of the 185 study participants.

Characteristics	Smear-negative presumptive pulmonary TB cases			p-value
	All patients (n=185)	Culture positive (n=19)	Culture negative (n=166)	
Age, median (IQR)	38 (23-55)	32 (21-47)	40 (26-56)	0.27
Male gender	114(61.6%)	14(73.7%)	100(60.2%)	0.25
Taking antibiotics	106(57.3%)	11(58%)	95(57.2%)	0.95
TB contact history	38 (20.5%)	6(31.6%)	32(19.3%)	0.21
Presenting TB symptom				
Cough >4 weeks	93(50.3%)	13(68.4%)	80(48.2%)	0.09
Chest pain	134(72.4%)	16(84.2%)	118(71%)	0.22
Night sweats	131(70.8%)	14(73.7%)	117(70.5%)	0.77
Fever	75(40.5%)	9(47.4%)	66(39.8%)	0.52
Weight loss	111(60%)	15(79%)	96(58%)	0.07
Shortness of breath	123(66.5%)	12(63.2%)	111(67%)	0.75
Loss of appetite	129(69.7%)	15(79%)	114(68.7%)	0.35

Abbreviations: TB=tuberculosis; IQR= interquartile range

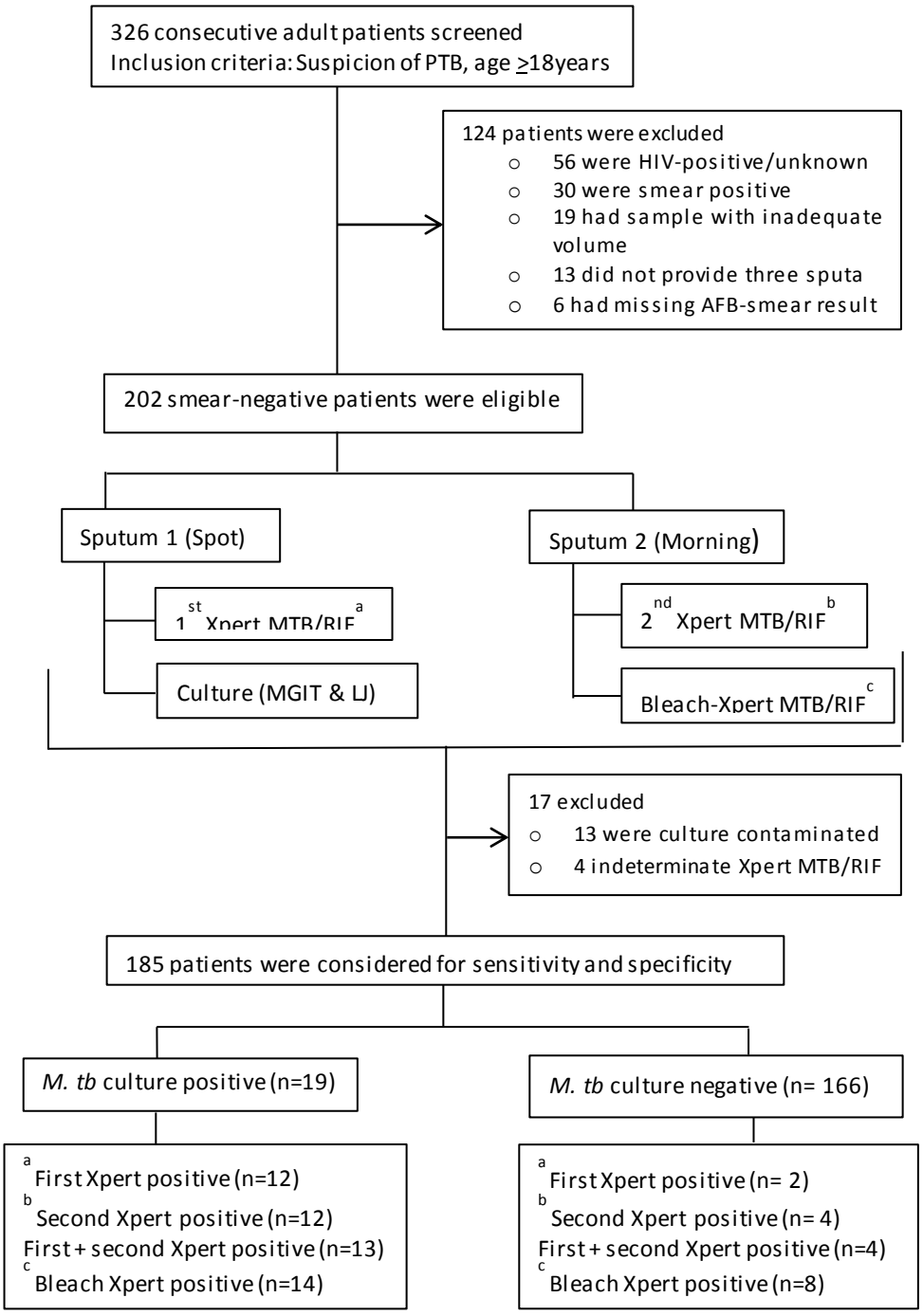

Figure 1. Overall study flow diagram explaining participants' recruitment, sample processing & diagnostic test results. **Key**: MGIT, Mycobacteria growth indicator tube: L-J, Löwenstein-Jensen: TB, tuberculosis: *M. tb*, *Mycobacterium tuberculosis.* [a] Xpert test on spot sputum: [b] Xpert test on morning sputum: [c] Xpert test on bleach-treated and concentrated sputum.

3.4.2. Diagnostic accuracy of direct Xpert

Nineteen (10.3%) of 185 smear-negative presumptive cases had culture (LJ and/or MGIT) confirmed TB. Among the 185 screened, 7.6% tested positive on the first Xpert and an additional 1.6% on the second Xpert, if no pre-test bleach treatment was performed. Using mycobacterial culture as the reference standard, the sensitivity of Xpert in spot and morning sputum was the same [63.2% (95%CI: 41.5-84.8)]. However, when results of both spot and morning Xpert were considered together, the sensitivity [68.4% (95%CI: 47.5-89.3)] slightly increased compared with either test alone, though the difference was not statistically significant (p-value = 0.76). Among 166 culture-negative cases, 2 spot and 4 morning sputa were Xpert-positive. The estimated specificity of Xpert was 98.8% in spot and 97.6% in morning sputum samples. A summary of the overall Xpert performance is depicted in **Table 3.**

3.4.3. Performance of Xpert on bleach-concentrated sputum

Twenty two (12%) of the 185 patients with smear-negative sputum were positive using bleach-concentrated Xpert, with 8 of them (36.4%) being culture-negative. The sensitivity of a single direct Xpert for culture-confirmed tuberculosis was 63.2% (95%CI: 41.5–84.8) and rose to 73.8% (95%CI: 58.6–97) for bleach-treated samples, which is also higher than the sensitivity when testing two samples by direct Xpert ([68.4% (95%CI: 47.5-89.3)]. Bleach-concentration and pelleting allowed an additional 7 cases of TB (missed on the first and second direct Xperts) to be detected, 5 of which were from culture-negative cases.

The specificity of Xpert was not significantly affected by bleach-concentration and pelleting: 95.1% (95%CI: 92-98.4) for bleach-concentrated sputum versus 98.8% (95%CI: 97-100) and 97.6% (95%CI: 95-100) for direct Xpert in spot and morning sputa (p-value 0.92). However, the positive predictive value was lower for bleach-processed sputum [63.6% (95%CI: 43.5-83.7) as compared to unprocessed spot [85.7% (95%CI: 63.4-100)] and morning [75% (95%CI: 53.8-96.2)] sputa (data not shown). There were 5 patients, whose cultures grew NTM. One of these patients had a positive Xpert after bleach-concentration and pelleting. There was no rifampicin resistance detected in any of the sputum samples tested by Xpert.

Table 3. Diagnostic performance of Xpert MTB/RIF assay compared to culture as reference standard, stratified to the type of sample or number of samples tested per patient.

	Reference standard		Sensitivity (95%CI)	Specificity (95%CI)
Index test (Xpert MTB/RIF)	**Positive**	**Negative**		
One spot sputum				
Positive	12	2	63.2%(41.5-84.8)	98.8%(97.1-100)
Negative	7	164		
One morning sputum				
Positive	12	4	63.2%(41.5-84.8)	97.6%(95.3-99.9)
Negative	7	162		
Bleach-concentrated pellet				
Positive	14	8	73.8%(53.8-93.5)	95%(91.9-98.4)
Negative	5	158		
Two sputa (spot + morning)				
Positive	13	4	68.4%(47.5-89.3)	97.6%(95.3-99.9)
Negative	6	162		
Three sputa (2 direct + 1 pellet)				
Positive	15	9	78.9%(60.6-97.3)	94.6%(91.1-98.0)
Negative	4	157		

M. tuberculosis culture positivity was used as reference standard, defined as identification of *M. tuberculosis* in at least one positive standard sputum culture (either on LJ slopes or MGIT culture). CI= confidence interval

3.4.4. Indeterminate Xpert results

Higher numbers of invalid Xpert results were observed when using bleach-concentrated sputum samples compared to unprocessed samples [1.5% (3/202) versus 0.3% (1/404)]. Overall, Xpert was indeterminate in 4 of 606 tests performed (0.6%), a rate that was much lower than the overall culture-contamination rate of 5% (20 of 404 cultures, 7 on both media, 4 only in MGIT and 2 only on LJ). Three Xpert-indeterminate samples were culture-negative and one was culture-positive.

3.5. Discussion

Smear-negative pulmonary TB constitutes a major burden of undiagnosed TB in resource poor settings [7]. It is associated with poor treatment outcomes, including death due to delayed diagnosis or non-diagnosis [3, 7]. Multiple studies have consistently shown that Xpert can identify a substantial proportion of smear-negative TB patients, particularly in HIV co-infected

patients [9, 12, 18]. There is a paucity of data on the diagnostic accuracy of the Xpert in smear- and HIV-negative patients in areas of high TB prevalence such as Ethiopia.

Our study suggests that application of Xpert on a single smear-negative sputum specimen can substantially increase the yield of confirmed TB cases. This is consistent with those reported by others regarding the effectiveness of Xpert in accurately detecting the presence of *M. tuberculosis* in smear-negative specimens [18, 19]. The observed sensitivity of a single Xpert (63.2%) for smear-negative TB is comparable with reported sensitivities ranged from 61% - 71.7% [13, 18, 20, 21]. Repeating Xpert test using a different sputum specimen per patient insignificantly increased the sensitivity. In high TB prevalence settings such as Ethiopia, repeating Xpert test may increase laboratory workload and expenses, though further study is warranted to ascertain the incremental yield from the second Xpert test.

There is no published data to the best of our knowledge that evaluated the performance of Xpert using bleach-concentrated sputum samples. Previous studies demonstrated non-significant improvement in sensitivity of Xpert when sputum samples were centrifuged [22, 23]. We documented an increased sensitivity by simple bleach-concentration and pelleting of sputum samples, allowing detection of 7 cases who would have been missed by two direct Xperts. Similar to most previous studies performed the specificity was high for Xpert, ranging from 97-100% [13, 23, 24]. The difference in specificity of Xpert after bleach-concentration and pelleting was not statistically significant from direct testing. Sodium hypochlorite (NaOCl) is cheap and available almost anywhere as household bleach. As a potent disinfectant, NaOCl also has the advantage of limiting the risk of laboratory infection. In addition, the relative centrifugal force needed for concentration of mycobacteria can easily be achieved by low cost table top centrifuge affordable under existing conditions of most TB laboratories in developing countries equipped with an Xpert device. On the other hand, bleach-concentration and pelleting increased the rate of Xpert indeterminate test results. This might be due to the sputum being over concentrated after centrifugation, generating viscous mixture debris and the NaOCl blocking the channels in the cartridge.

In our study, Xpert on bleach-concentrated sputum detected 8 cases from smear-negative TB suspects which were not picked up by either of the culture methods. Similarly, in the study by Rachow and colleague [18], 9% of culture-negative but Xpert-positive patients were classified

as clinical TB cases, with documented positive response to anti-TB treatment. Unfortunately, due to logistic constraints, the 8 cases in our study were not followed. As it is of paramount importance to ascertain that these cases, detected only by Xpert in bleach-concentrated sputum are unambiguously true TB cases, a more thorough clinical evaluation study which specifically addresses such cases is warranted. Among culture positive cases 4 were identified as NTM using SD Bioline MPT64 TB Ag test, one of which was positive on Xpert after bleach-concentration. Previous studies by Moure *et al*.[13] and Marlowe *et al.* [25] reported 100% specificity of Xpert with 20 and 41 NTM samples, respectively. In the current study, due to limited resources, differentiation of NTM from MTBC was done by SD Bioline MPT64 TB Ag test. It is possible that due to low MPT64 antigen expression by some lineage of MTBC, the SD Bioline MPT64 TB Ag test can be false negative. If this is assumed true, the specificity of Xpert for NTM would be 100% in our study.

We documented a moderate sensitivity (63.2%) of single direct Xpert among smear-negative culture-positive cases. Xpert identified majority of the culture confirmed cases with high specificity. This gives the clinician sufficient confidence to initiate anti-TB treatment when Xpert is positive. A negative Xpert does not exclude a diagnosis of smear-negative TB given the fact that the test was unable to identify 36.8% of patients with culture confirmed TB. Patients with a high clinical probability of TB despite a negative Xpert should be started on anti-TB treatment. It is important to ensure that clinicians are aware of the Xpert limitations prior to its implementation. Despite this, Xpert has distinct advantages over culture; a faster turn-around time, providing same-day diagnosis which could potentially limit loss to follow up during diagnostic evaluation of smear-negative TB patients.

This study has some limitations. First, we did not document treatment outcome of the patients. It is not clear how many of the patients testing positive for *M. tuberculosis* by Xpert in this study would have been started on TB treatment based on the clinicians' decision if Xpert testing was not available. Among culture- and Xpert-negative cases there may be false-negative cases that started anti-TB treatment on clinical grounds and improved, cases that were most likely true TB. Secondly, all sputum samples received NALC-pre-treatment which could have some effect on direct and bleach-concentrated Xpert result. Lastly, although the sensitivity of the Xpert in this study was improved on bleach-concentrated sputum sample

compared to direct sputum, we had insufficient power to determine whether this difference was statistically significant.

In conclusion, our results suggest that direct Xpert can rapidly diagnose TB in smear-negative patients with modest sensitivity and excellent specificity. Smear-negative TB patients could benefit from Xpert particularly in those areas where no culture is available. Testing of single sputum by Xpert can reach reasonable sensitivity and results would be available on the same day, avoiding loss of patients and treatment delay. Moreover, bleach-concentration and pelleting of sputum samples improves the sensitivity of Xpert, probably without affecting the specificity significantly. Further prospective studies including clinical outcome data (such as response to treatment) with larger sample size are required to clarify these findings.

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Chapter four: Paper II

4. Concentration of lymph node aspirate improves the sensitivity of acid fast smear microscopy for the diagnosis of tuberculous lymphadenitis in Jimma, Southwest Ethiopia

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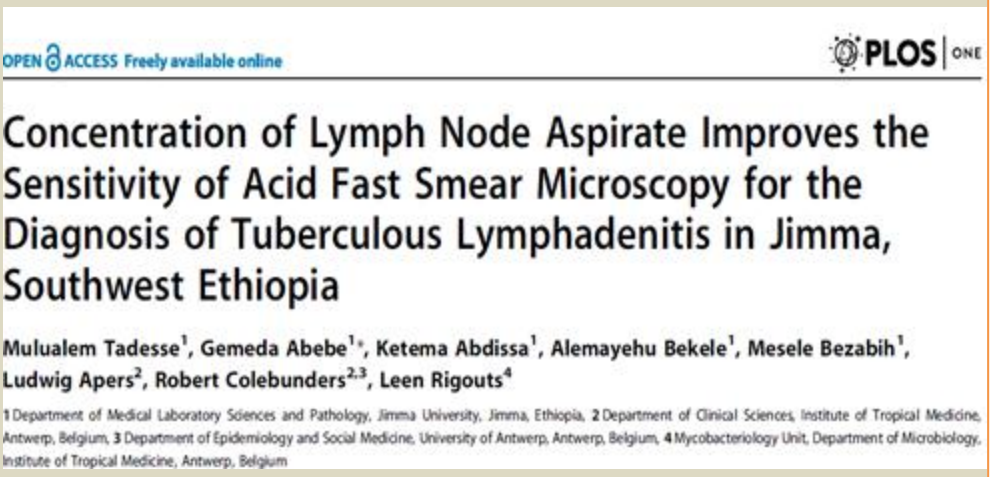

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Concentration of Lymph Node Aspirate Improves the Sensitivity of Acid Fast Smear Microscopy for the Diagnosis of Tuberculous Lymphadenitis in Jimma, Southwest Ethiopia

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4.1. Abstract

Background: Tuberculous lymphadenitis (TBLN) is the most common form of extrapulmonary tuberculosis. The cytomorphological features of lymph node smears have reduced specificity for the diagnosis of tuberculosis. The diagnosis of TBLN with direct smear microscopy lacks sensitivity due to the limited number of bacilli in lymph node aspirate. Therefore, we aimed to assess whether the concentration of lymph node aspirate improves the sensitivity of acid fast smear microscopy for the diagnosis of tuberculous lymphadenitis.

Methods: A cross-sectional comparative study was conducted on 200 patients clinically suspected for tuberculous lymphadenitis in Jimma, Ethiopia. Lymph node aspirate was collected. The first two drops were used for cytomorphological study and direct acid fast staining. The remaining aspirate was treated with N-acetyl-L-cysteine (NALC) and concentrated by centrifugation at 3000 g for 15 minutes. The sediment was used for acid fast staining and culture. Differentiation of *M. tuberculosis* complex (MTBC) from non-tuberculous mycobacteria (NTM) was done by para-nitrobenzoic acid susceptibility test.

Results: Complete data were available for 187 study subjects. 68% (127/187) were positive for *M. tuberculosis* on culture. Four isolates, 2.1% (4/187), were identified as NTM. The detection rate of direct smear microscopy was 25.1% and that of the concentration method 49.7%. Cytomorphologically, 79.7% of cases were classified as TBLN. The sensitivity of direct smear microscopy was 34.6%, for concentrated smear microscopy 66.1%, and for cytomorphology 89.8%. Two AFB positive cases on concentration method were non-tuberculosis mycobacteria (NTM). The concentration method yielded a positive result from seven cases diagnosed as suppurative abscess by cytology. Both for the direct and concentration methods the highest rate of AFB positivity was observed in smears showing caseous necrosis alone. Smear positivity rate decreased with the appearance of epithelioid cell aggregates.

Conclusion: The concentration of lymph node aspirates for acid fast smear microscopy had significantly higher sensitivity than direct microscopy.

4.2. Introduction

Tuberculosis (TB) is a global health burden especially in the developing world [1]. Ethiopia ranks 8th among the 22 high TB burden countries and third in terms of the number of extrapulmonary tuberculosis (EPTB) cases. The prevalence of all forms of TB is estimated at 261 per 100,000 population, leading to an annual mortality rate of 64 per 100 000 [2].

In Ethiopia, EPTB accounted for 34.8% of TB cases with the largest group being tuberculous lymphadenitis (80%) [3]. Definitive diagnosis of tuberculous lymphadenitis is often difficult as most of the available techniques are low either in sensitivity or specificity as compared to culture. Clinical features, though indicative of tuberculous etiology, are not adequate for making a definitive diagnosis [4]. Fine needle aspiration cytology (FNAC) is a simple and rapid diagnostic technique but is characterized by low specificity [5]. Direct smear microscopy is the cornerstone for diagnosis of pulmonary TB in developing countries. It is rapid, inexpensive, specific, and capable of identifying the most infectious cases of TB, but its sensitivity in EPTB cases is limited to 20–43% [6,7,8]. Mycobacterial culture is the gold standard method for detection of tubercle bacilli with the sensitivity ranging from 70% to 80% [7], but it is time-consuming and requires specialized safety procedures as it is performed in a biosafety level -2 facility.

Studies have shown that the sensitivity of smear microscopy can be improved if the sputum sample is liquefied with one or more chemical reagents and then concentrated by centrifugation before acid fast staining [6,7,8,9]. N-acetyl-L cysteine (NALC) is used for the treatment of sputum for culture. This study aimed to assess whether treatment with NALC followed by concentration improves the sensitivity of smear microscopy of lymph node aspirate. The basic assumption was that the NALC powder in this solution liquefies the specimen and lyses the cells, including the ones containing the bacilli, after which the solution can be further concentrated by centrifugation. To the best of our knowledge no study so far has evaluated a concentration method to detect mycobacterial bacilli in lymph node aspirates in Ethiopia.

4.3. Methods

4.3.1. Recruitment of study participants

A cross-sectional comparative study was conducted at Jimma University Specialized Hospital from April1, 2012 to September 30, 2012. A total of 200 clients with suspected TBLN and subjected to fine needle aspirate were included in the study. Patients on anti-tuberculosis treatment at the time of the lymph node aspiration were excluded from the study. Demographic and clinical information of the participants was collected by trained clinical nurses using a pre-tested questionnaire. HIV test results were collected from the medical records after obtaining written consent from the clients.

4.3.2. Collection of lymph node aspirate

Lymph node aspirate was collected after receiving written consent from the study participants. Briefly FNA was performed from a swollen superficial lymph node by using a sterile 21-gauge needle with an attached syringe. The overlying area was cleaned with 70% alcohol. Then the node was punctured by developing a negative pressure in the syringe. Multiple (average six) in and out passes were made by the needle without exiting the node. After removing the needle drops of aspirate were placed on two clean slides for FNA cytology and direct AFB smear. The rest of the specimen was transferred into a falcon tube containing sterile normal saline for concentrated ZN staining and culture at Jimma University Laboratory of Mycobacteriology.

The gross appearance of aspirate was noted as caseous for cheese like or yellow-white aspirate and purulent/non-caseous for greenish yellow or yellow aspirate.

4.3.3. Fine needle aspiration cytology (FNAC)

FNA smears were prepared on clean slides on the spot. The slides were air dried and flooded with freshly filtered Wright's stain and buffered with clean tap water. The buffered slides were continuously stained with Wright's stain for 10 minutes, washed with tap water and air dried. Finally, the slides were examined by a pathologist to evaluate whether the morphology was suggestive for tuberculous lymphadenitis or not.

Cytological examination of FNA smears were considered diagnostic of TBLN in the presence of the following cytomorphological circumstances: epithelioid cell aggregate with or without Langerhans giant cells and necrosis, epithelioid cell aggregate without necrosis, necrosis without epithelioid cell aggregate or polymorphocytes with necrosis [10].

4.3.4. Direct smear microscopy for the detection of AFB

Immediately after the specimen was collected, a drop of aspirate was placed on a clean slide to make a direct smear. The standard Ziehl-Neelsen staining procedure was applied [2]. Stained smears were examined for the presence of AFB under oil-immersion (1000x) using a light microscope.

4.3.5. Concentration method for detection of AFB

FNA specimen was first decontaminated and digested with a 1% NALC-4% sodium hydroxide (NaOH)-2.9% sodium citrate solution and concentrated by centrifugation at 3000 g for 15 minutes at Jimma University Laboratory of Mycobacteriology [2]. After centrifugation the supernatant was decanted carefully and 2–3 drops of the sediment were placed on a clean slide and stained with Ziehl-Neelsen (ZN) acid fast staining. The remaining sediment was used for culture as described below. All AFB smear positive slides were graded based on the IUATLD scale [11].

4.3.6. Mycobacterial culture

The processed specimens were resuspended by 1 ml of phosphate buffer and inoculated on two L-J tubes. The inoculated L-J tubes were incubated at 37uC and examined daily for the first week for any contamination. For the next 7 weeks LJ media were observed once a week for the growth of mycobacteria. Growth of the mycobacteria was confirmed by visual inspection of colony morphology and microscopic examination of the colonies for acid fast bacilli (AFB). Differentiation of M. tuberculosis complex (MTBc) from non-tuberculous mycobacteria (NTM) was done by para-nitrobenzoic acid susceptibility test at 500mg/ml. A known ATCC strain of H37Rv was used as a positive control. Random slants of LJ media were inoculated with sterile distilled water with each run as a negative control.

4.3.7. Data analysis

Data were analyzed with SPSS version 16.0. Descriptive statistics were used for analysis of socio-demographic and clinical characteristics. Sensitivity, specificity, positive and negative predictive values of diagnostic methods were computed by using culture as gold standard method. A confirmed TBLN case was defined when growth was observed on culture, ZN staining confirmed AFB from the growth and if no growth was observed on LJ media containing PNB.

The association between the efficiency of the test method and factors affecting the efficiency were analyzed using binary logistic regression analysis by calculating odds ratios (ORs) with 95% confidence intervals. P-values of <0.05 were considered statistically significant.

4.3.8. Ethical clearance

Ethical clearance was first obtained from the Jimma University ethical review board. A letter of permission to conduct the study was obtained from Jimma University Specialized Hospital clinical director office. All patients or guardians in case of children were requested for written consent prior to enrolment to the study. Any information concerning the patients was kept confidential. Laboratory results were reported back to the physicians for treatment initiation or decision as early as available.

4.4. Result

4.4.1. Characteristics of study participants

Thirteen samples from 200 patients with presumptive TBLN that submitted a lymph node aspirate had a contaminated culture result. Thus, only 187 suspects were included in this analysis. Out of 187 suspected patients, 54.4% (102/187) were females. The age range of the suspects was between 3 and 78 years with a mean age of 29.5(614) years. Half, [49.7% (93/187)], of the study participants were within the 15 to 30 years age group.

The majority (58.3% (109/187)) of clients with presumptive TBLN presented with cervical lymphadenopathy. The mean duration of lymph node enlargement was 11 months. The presence of a lymph node scar was observed in 35.8% (66/187) of cases. Lymph node aspirate appeared purulent in 57.2% (107/187) of the cases. HIV test result was known for only 58.3% (109/187) of suspects; of whom 8.2% (9/109) were HIV positive.

4.4.2. Detection rate

Out of 187 FNA specimens processed and inoculated on L-J media, M. tuberculosis complex was isolated in 68% (127/187) and NTM in 2.1% (4/187) of the cases. Thus, TBLN was confirmed in 68% (127/187) of the suspected cases. On FNAC 79.7% (149/187) of cases were cytomorphologically diagnosed as TBLN. AFB were detected in 25.1% (47/187) of the suspected cases by direct ZN staining and in 49.7% (93/187) of the suspected cases with the concentration method (P,0.0001). The concentration method detected 46 extra patients with an incremental yield of 24.7% (46/187).

4.4.3. Sensitivity, specificity, positive and negative predictive values

Among 127 culture-positive samples, 44(34.6%) were AFB positive on direct ZN stain and 84(66.0%) on NALC-NaOH concentration method. Out of 56 culture negative samples, only one was positive on direct smear and 7(12.5%) on NALC-NaOH concentration method. Similarly, on FNA cytology 90.5% (115/127) of culture positive cases showed cytomorphological features consistent with TB. From culture negatives, 55.4% (31/56) were classified as TB by FNA cytology. Against culture, direct smear microscopy showed sensitivity of 34.6% and specificity of 98.2%, positive predictive value of 93.6%, and negative predictive value of 39.3%. The NALC-NaOH concentration method had 66.0% sensitivity, 87.5% specificity, 90.5% positive predictive value, and 52.3% negative predictive value. Cytology had sensitivity of 90.5%, specificity of 44.6%, positive predictive value of 77.2%, and negative predictive value of 65.8% (Table 1).

Table.1: Sensitivity, specificity, PPV*, NPV and test efficiency of direct ZN stain, concentration method and cytology against culture (n=187).**

Methods	Sensitivity	Specificity	PPV	NPV	Test efficiency
Direct ZN staining	34.6%	98.2%	93.6%	39.3%	53.0%
Concentration method	66.0%	87.5%	90.5%	52.3%	71.0%
Cytology	90.5%	44.6%	77.2%	65.8%	74.8%

*PPV=positive predictive value, **NPV= negative predictive value

4.4.4. Density of acid-fast bacilli (AFB)

On direct ZN method, a total of 47 cases were AFB positive and the majority of them (55.3% (26/47)) were scanty, 29.8% (14/47) cases 1+, 10.6% (5/47) cases 2+ and only 2 cases were graded as 3+. Among 93 AFB positive cases on concentration method, 23.7% (22/93) of cases graded scanty, 43.0% (40/93) were grade 1+, 25.8% (24/93) of cases graded 2+, and 7.5% (7/93) of cases graded 3+.

Among 26 smears which were graded as scanty by the direct method, 14 were increased to 1+ and 9 to 3+ by the concentration method. Of 14 smears graded as 1+ by the direct method, 4 remained 1+ and 9 increased to 2+ by the concentration method (**Table 2**). Among 46 specimens that were AFB positive only after concentration, 47.8% (22/46) had grade of 1+, 41.3% (19/46) cases grade of scanty and 10.9% (5/46) cases grade of 2+.

Table-2: Incremental yield of AFB grading on smears prepared after concentration as compared to direct smear microscopy at JUSH, Jimma, South West Ethiopia (n=47).

	Grade on concentration method				
Grade on direct ZN	**Scanty**	**1+**	**2+**	**3+**	**Total**
Scanty	3	14	9	0	26
1+	0	4	9	1	14
2+	0	0	1	4	5
3+	0	0	0	2	2
Total	3	18	19	7	47

4.4.5. Cytomorphological features

Hundred forty nine (79.7%) were classified as TBLN on cytology. Out of these, the concentration method detected AFB in 55.7% (83/149) of the cases versus 28.9% (43/149) by direct smear. Similarly, on the culture, mycobacteria were isolated in 77.2% (115/149) of cases diagnosed as TBLN on cytology. The concentration method detected AFB from 10 cases which were missed on cytology: 7 from suppurative abscesses and 3 from pyogenic infections (**Table 3**).

Table.3: Comparisons of cytomorphologic features with AFB positivity on direct and concentrated method of Z-N staining (N=187).

FNAC result	**Total cases**	**Direct ZN staining**		**Concentrated ZN staining**	
	% (n/N)	Positive %(n/N)	Negative %(n/N)	Positive %(n/N)	Negative %(n/N)
TBLN	79.7(149/187)	28.9(43/149)	71.1(106/149)	55.7(83/149)	44.3(66/149)
Suppurative abscess	7.0(13/187)	30.8(4/13)	69.2(9/13)	53.8(7/13)	46.2(6/13)
Pyogenic infection	5.3(10/187)	0.0(0/10)	100(10/10)	30.0(3/10)	70.0(7/10)
Reactive LN*	4.3(8/187)	0.0(0/8)	100(8/8)	0.0(0/8)	100(8/8)
Other diagnosis	3.7(7/187)	0.0(0/7)	100(7/7)	0.0(0/7)	100(7/7)

*Reactive LN= Reactive lymphadenitis

Detailed cytomorphological features of tuberculous lymphadenitis were clearly described for 82 cases. Out of these, 39.0% (32/82) showed epithelioid cell aggregate with necrosis followed by necrosis without epithelioid cells in 36.6% (30/82), epithelioid cell aggregate without

necrosis in 12.2% (10/82) and polymorphocytes with necrosis in 12.2% (10/82) cases. Out of 30 cases with caseous necrosis without epithelioid cells, AFB was present on direct smear in 56.7% (17/30) of them and in 73.3% (22/30) by the concentration method. While of 10 cases showing epithelioid cell aggregates with necrosis, the AFB positivity rate was 1 by direct ZN staining and 4 by concentration method. AFB positivity both by direct and concentration method increased with the presence of necrosis alone and a lower rate of AFB positivity was observed with the presence of epithelioid cell aggregates alone (**Table 4**).

Table-4: Correlation of cytomorphologic features of TBLN with AFB positivity (direct &concentrated smear) and culture positivity (N=82).

Cytological category	Cases %(n/N)	Direct ZN positive %(n/N)	Concentrated ZN positive %(n/N)	Culture positive %(n/N)
Epithelioid cell with necrosis	39.0%(32/82)	37.5%(12/32)	53.1%(17/32)	68.8%(22/32)
Epithelioid cell without necrosis	12.2%(10/82)	10.0%(1/10)	40.0%(4/10)	70.0%(7/10)
Necrosis without epitheloid cell	36.6%(30/82)	56.7%(17/30)	73.3%(22/30)	86.7%(26/30)
Polymorphs with necrosis	12.2%(10/82)	50.0%(5/10)	60.0%(6/10)	100%(10/10)

4.4.6. Factors assessed for association with smear positivity on concentration method

Smear positivity by the concentration method was statistically associated with the presence of a lymph node scar [p-value = 0.003, OR=2.5, 95%CI= 1.3–4.6]. Aspirates with purulent appearance were 2 times more likely to be positive on direct smear microscopy [p-value = 0.037, OR= 2.01, 95%CI = 1.0–4.3]. However, the AFB detection rate with the concentration method did not vary with the nature of the aspirate (p-value = 0.82) (**Table 5**).

Of the 9 HIV positive patients, two were positive on direct ZN method and five on concentration method. In HIV infected individuals, the detection rate of ZN staining increased from 22.2% by direct ZN method to 55.6% by concentration method.

Table-5: Factors assessed for associated with smear positivity when considering concentration method alone in Jimma, southwest, Ethiopia.

Factors		Concentration method (ZN)		OR[95%CI]	P-value
		Positive	Negative		
Sex	Male	57.6%(49/85)	42.4%(36/82)	1.8[1.0-3.4]	
	Female	43.1%(44/82)	56.9%(58/82)	1.0	0.20
Age	0-15	48.1%(13/27)	51.9%(14/27)	1.8[0.5-6.0]	
	15-30	47.3%(44/93)	52.7%(49/93)	1.8[0.7-5.1]	
	30-45	61.7%(29/47)	38.3%(18/47)	3.2[1.1-9.9]	0.51
	>45	35.0%(7/20)	65.0%(13/20)	1.0	
HIV	Positive	55.6%(5/9)	44.4%(4/9)	1.1[0.5-1.9]	
	Negative	50.0%(50/100)	50.0%(50/100)	1.0[0.3-4.3]	0.924
Lymph node	Unknown	48.7%(38/78)	51.3%(40/78)	1.0	
	Cervical	49.5%(54/109)	50.5%(55/109)	0.97[0.30-3.06]	0.600
	Axillary	48.6%(18/37)	51.4%(19/37)	1.00[0.28-3.60]	
	Inguinal	64.3%(9/14)	35.7%(5/14)	2.10[.40-11.3]	
Duration of LN swelling	≤5 months	48.7%(55/103)	51.3%(58/103)	1.05[0.37-2.99]	0.360
	6-10months	62.5%(20/32)	37.5%(12/32)	1.88[0.54-6.62]	
	11-15months	39.1%(9/23)	60.9%(14/23)	0.52[0.14-1.97]	
	>15 months	47.4%(9/19)	52.6%(10/19)	1.00	
Lymph node mobility	Yes	52.3%(57/109)	47.7%(52/109)	1.48[0.77-2.83]	0.353
	No	45.3%(34/75)	54.7%(41/75)	1.00	
Lymph node scar	Present	63.6%(42/66)	36.4%(24/66)	2.52[1.30-4.89]	0.003
	Absent	42.1%(51/121)	57.9%(70/121)	1.00	
Gross specimen appearance	Purulent	50.5%(54/107)	49.5%(53/107)	0.94[0.50-1.76]	0.816
	Caseous	48.8%(39/80)	51.2%(41/80)	1.00	

4.5. Discussion

In developing countries, like Ethiopia, the conventional diagnostic tool for TBLN mainly relies on FNA cytology and direct smear microscopy. FNAC has limited specificity because of the presence of cytologic components such as epithelioid cells aggregates, multinucleated giant

cells, and necrotic material in lesions other than those associated with TB such as fungal infections, other inflammatory conditions and sarcoidosis [5,6]. Moreover, FNA requires highly trained pathologist which is the case in Ethiopia who are not available at relatively lower level health facilities. Culture is the gold standard for the diagnosis of TBLN. However, the availability and affordability of this method in resource limited settings like Ethiopia necessitates the quest for other techniques with added value over direct Z-N microscopy. In such settings concentrating FNA sample can improve the diagnosis of TBLN.

The low sensitivity (34.6%) of direct smear examination in our study is in agreement with reports from other studies [6,12]. The nature of specimen and the scanty bacilli found in the lymph node aspirates could be the main factor for the decreased sensitivity of direct smear.

Many reports have shown that liquefaction of clinical samples followed by centrifugation significantly increases the smear sensitivity up to 72% [6,13]. Our findings demonstrated comparable results. The sensitivity of AFB smear on direct Z-N was 34.6% and it increased to 66.0% on concentration method. The increased smear positivity by the concentration method is attributable to the higher density of bacilli per microscopic field and reduction of debris, leaving a clear field for microscopy.

Seven positive cases on concentration method were culture negative. This may be due to the fact that AFB positivity rate on the concentration method was highest when the cytomorphological feature was caseous necrosis. A crucial phenomenon happens within the caseous lesion is the death of the majority, if not all, of the tubercle bacilli. These non-viable bacilli are unable to grow on culture. Cytomorphological features should be used in conjunction with concentration method to manage such cases in clinical practice.

Our results showed that the majority of positive cases (55.5%) on direct Z-N staining had scanty AFB grade and searching for them was time consuming and tedious. On the concentration method, 76.4% of positive cases showed grades of AFB positivity above scanty, making them easily visible and detectable. Other studies also reported that the AFB positivity grade was higher by the concentration method making the bacilli easily visible within a shorter screening time [6,13].

AFB positivity rate by direct and concentration method was highest when the cytomorphological features of FNAC was necrosis without epithelioid cell aggregates. By the concentration method, there is an increase in AFB smear positivity from 40.0% in those with epithelioid cell aggregates alone to 53.0% when epithelioid cell aggregates present with necrosis and to 73.3% when necrosis without epithelioid cell aggregates were seen. Similarly, Bezabih et al. [14] and Gupta et al. [10] observed the highest rate of AFB positivity in smears showing necrosis alone and decreased smear positivity rates with the appearance of epithelioid cell aggregates. This is probably due to the fact that the central necrotic portion of the tubercle contains more bacilli.

Out of 13 cases classified as suppurative abscess on cytology, TBLN was diagnosed in 7 cases by the concentration method and in 4 cases by direct smear microscopy. The possible explanation for the misdiagnosis of specimens as suppurative abscess on cytology may be the absence of characteristic features within abundant mixed inflammatory super infections by other bacteria.

In the current study TBLN suspects with lymph node scar were 2.5 times more likely to yield positive result on the concentration method as compared to those patients without scar. During infection of lymph node, the caseous material perforates the deep fascia and escapes into the superficial fascia resulting in collar stud abscess formation, the abscess may present with persistent discharging sinus and finally developed in scar.

4.6. Conclusion

In conclusion, the sensitivity of the concentration method was significantly higher in comparison with the direct smear microscopy. In addition, the majority of positive cases by the concentration method showed a high grade of AFB positivity, making the screening process easier, faster and less laborious. The highest AFB positivity rate was observed in cytomorphological features consistent with caseous necrosis. Finally we suggested that in addition to routine cytology the concentration method can increase the diagnostic yield for TB lymphadenitis.

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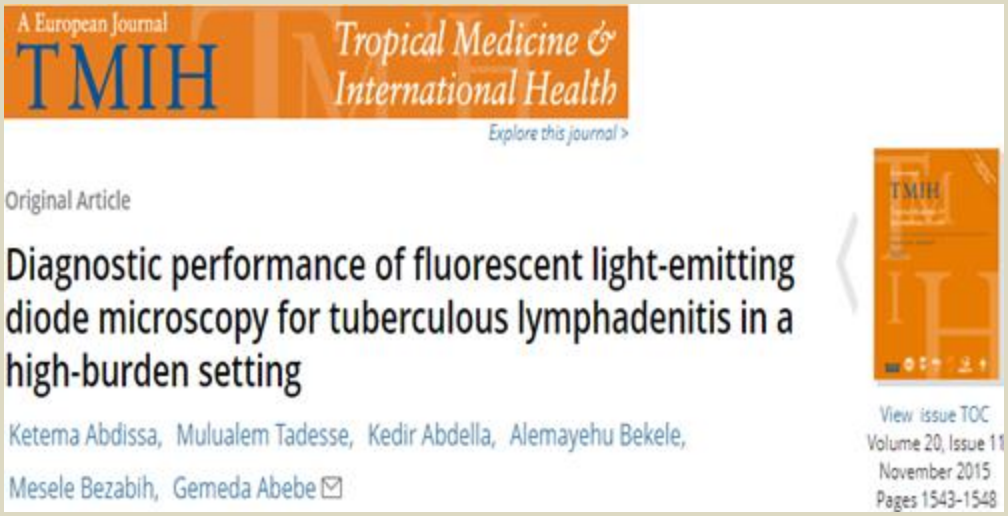
Chapter five: Paper III

5. Diagnostic performance of fluorescent light emitting diode microscopy for tuberculous lymphadenitis in a high burden setting

This chapter is published as



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5.1. Abstract

Background: Diagnosis of tuberculous lymphadenitis using fine-needle aspiration cytology is a simple and safe but low-specificity method, whereas conventional smear microscopy has variable sensitivity due to low bacterial load. We evaluated the diagnostic performance of fluorescent light-emitting diode (LED) microscopy on routinely collected fine-needle aspirates from tuberculous lymphadenitis presumptive cases.

Methods: Fine-needle aspirates were collected from patients clinically suspected of having tuberculous lymphadenitis as part of routine diagnosis. Smear preparation was performed from the aspirate and processed for cytology, conventional Ziehl–Neelsen and LED microscopy. The remaining aspirate was processed for culture on Lowenstein–Jensen media. Capilia TB-Neo test was used to differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria.

Results: A total of 144 tuberculous lymphadenitis presumptive cases were included. 66.7% (96/144) were positive for *M. tuberculosis* complex on culture. Only one isolate was identified as nontuberculous mycobacteria. The detection rates of Ziehl–Neelsen and LED microscopy were 18.8% (27/144) and 34% (49/144), respectively. As compared to culture, sensitivity was 25.0% [95% CI: 16.3–33.7] for Ziehl–Neelsen microscopy and 45.8% [95% CI: 35.9–55.8] for LED microscopy. The specificity was 93.8% [95% CI: 86.9–100] for Ziehl–Neelsen microscopy and 89.6% [95% CI: 80.9–98.2] for LED microscopy. LED microscopy showed a statistically significant increase in sensitivity and similar specificity compared to Ziehl–Neelsen microscopy. Mean reading time of positive slides was 2.62 min/slide for Ziehl–Neelsen and 1.60 min/slide for LED microscopy. Cytology showed sensitivity of 82.3% and specificity of 54.2%. LED microscopy detected TB bacilli in 33.3% of cases cytologically classified as suppurative abscess.

Conclusion: The LED microscopy for tuberculous lymphadenitis had significantly higher sensitivity and shorter screening time than Ziehl–Neelsen microscopy. Use of LED microscopy among cases classified as suppurative abscess on fine-needle aspirate cytology improves evidence-based diagnosis of presumptive tuberculous lymphadenitis cases. Moreover, LED microscopy could be considered as an alternative approach in settings where fine-needle aspirate cytology is impractical.

5.2. Introduction

Extrapulmonary tuberculosis (EPTB), particularly tuberculous lymphadenitis (TBL), continues to be a major health problem in developing countries [1, 2]. In Ethiopia, EPTB with TBL is the most common type accounting for 80% of EPTB [1, 3]. However, EPTB as a much more obscure process is more difficult to diagnose than pulmonary TB. The differential diagnosis of EPTB is extensive and includes infections (viral, bacterial or fungal), and neoplasms (lymphoma or sarcoma, metastatic carcinoma) [2, 4, 5].

Ethiopian national guideline recommends use of fine needle aspiration cytology (FNA) and tissue biopsy as routine diagnostic methods among patients having high index of clinical suspicion for TBL [6]. However, the specificity is low due to the difficulty in distinguishing other granulomatous pathologies in the absence of acid-fast bacilli that result in the same cytological features. Other conventional diagnostic tools are neither sensitive nor specific to the diagnosis of TBLN [7, 8]. The conventional Ziehl–Neelsen (ZN) method is fast, cheap and widely used but lacks sensitivity, ranging from 20% to 43% [8–10]. Culture is the reference method for the detection and identification of tubercle bacilli, but may take 2–8 weeks to yield results and requires complex and specialized laboratory facilities [7–9]. Although molecular methods are rapid and show promising accuracy [11], they are costly and not available to be routinely used in developing countries like Ethiopia, where TBL is prevalent.

The sensitivity of smear microscopy is largely determined by the duration of microscopic examination [12, 13]. In high workload settings, the amount of time spent on examining each smear by conventional ZN is low which could probably compromise the sensitivity. LED microscopy allows a much larger area of the smear to be seen at a time and results in more rapid examination of the specimen [12, 14].

Conventional fluorescent microscopy has documented higher sensitivity than ZN and can reduce laboratory workloads [15], but uptake has been hampered by complexity of the microscope and mercury vapour lamp lighting system (short lifespan), the need for a dark room and perceived health risks associated with ultraviolet light exposure [16].
LED microscopy is a novel diagnostic tool developed primarily to provide resource-limited settings with access to the benefits of fluorescent microscopy. In comparison with conventional mercury vapour fluorescence microscopes, LED microscopes are less expensive,

the bulbs have a long half-life, do not pose the risk of releasing potentially toxic products and are reported to perform equally well in a light room [13, 14].

Based on findings from previous studies, WHO recommends conventional fluorescent microscopy be replaced by LED microscopy and that LED microscopy be phased in as an alternative for conventional ZN light microscopy for the diagnosis of pulmonary TB [13]. But there is limited information on the diagnostic performance of LED microscopy for the diagnosis of EPTB. In this study, we evaluated the diagnostic accuracy, incremental yield and the screening time of LED microscopy to diagnose presumptive TBLN.

5.3. Materials and methods

A prospective study was conducted in Jimma, south-west Ethiopia. A total of 154 presumptive TBLN cases were consecutively recruited into the study from April 2013 to August 2013. After written informed consent was secured, and FNA was collected from each presumptive TBL case by the pathologist in charge following the standard protocol. Macroscopic appearance of the aspirates was classified as follows: caseous, purulent or bloodstained.

Specimens were processed for conventional (ZN) light microscopy, LED microscopy and routine cytology. The remaining aspirate was resuspended in 1 ml sterile normal saline and processed for culture. Cytological examination was carried out in the pathology diagnostic unit by experienced pathologists. Briefly, smears were prepared directly from the aspirate. Air dried smears were stained with Wright's stain and examined by a pathologist. Cytological examinations were considered diagnostic of TB in the presence of the following cytomorphological appearances: epithelioid cell aggregate with or without Langerhans giant cells and necrosis, epithelioid cell aggregate without necrosis, necrosis without epithelioid cell aggregate or polymorphocytes with necrosis [17].

At Jimma University Mycobacteriology Research Center, the aspirate was used for AFB smear and culture. Two smears were made of each specimen. One was stained using the hot ZN method and examined under light microscopy (Olympus CX31 light microscope; Olympus, Tokyo, Japan) following the standard procedure [18]. The stained smears were examined for AFB under oil immersion (91000 magnification). At least 100 fields were examined before

reporting the smear as 'no acid-fast bacilli observed' [19]. The other smear was stained with auramine O and examined under LED microscopy (Primo Star iLED, Carl Zeiss, Gottingen, Germany) with 4009 magnification and 40 fields were examined. Briefly, the smears were covered completely with auramine O solution (Sigma-Aldrich, USA). After 20 min, the slides were washed with running water and decolorized by 0.5% acid alcohol solution for 3 min and counterstained with 0.5% potassium permanganate for 1 min. Stained smears were examined on the date of staining. Blind reading of the slide was performed by two independent laboratory technologists. All AFB smear-positive slides were graded based on the International Union Against Tuberculosis and Lung Diseases (IUATLD) scale. The time required to read individual slide was documented for all smear-positive slides.

For culture, the remaining aspirated specimen was resuspended in 1 ml sterile normal saline and transferred into 50-ml sterile falcon tube. An equal volume of standard decontaminating solution, a 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide (NaOH) and 2.9% sodium citrate solution, was added and centrifuged for 15 min at 3000 g. After centrifugation, the supernatant was decanted carefully and the sediment was resuspended by 1 ml of sterile phosphate-buffered solution (pH = 6.8). Finally, 100 µl of the sediment was used to inoculate on Lowenstein–Jensen (LJ) slants. A known ATCC strain of H37Rv was used as a positive control. Random LJ slants were inoculated with sterile phosphate-buffered solution with each run as a negative control. Differentiation of MTBC from NTM was performed by Capilia TBNeo test (TAUNS, Izunokuni, Japan). Colonies ≥1 were considered as culture positive.
Data were analyzed with SPSS version 16.0. Descriptive statistics were used for analysis of patient characteristics. Sensitivity, specificity, positive and negative predictive values with their corresponding 95% CIs were calculated for cytology, ZN and LED microscopy using culture as the reference standard. The detection yields of cytology, ZN and LED microscopy were also calculated per specimen.

The Ethical Review Committee of Jimma University approved the study. Patients signed informed consent form to participate in the study. Positive results were made available to clinicians for decision-making as early as available.

5.4. Result

One hundred and fifty-four presumptive TBLN cases were consecutively enrolled in the study. Of these, 10 were excluded from the final analysis due to contamination of the specimen on

culture (n = 7), smear result unavailable (n = 2) and NTM diagnosed (n = 1). Of the 144 presumptive TBL cases, 54.2% (78/144) were females. The mean age of clients was 27.4 (_14 SD) years. Majority of lymph node aspirates were obtained from lymphadenopathy of cervical region, which accounted for 69.4% (100/144). On gross examination, purulent aspirates were observed in 50.7% (73/144) of the cases, followed by caseous in 41.0% (59/144) and blood-stained aspirates in 8.3% (12/144) (**Table 1)**

Table-1: Socio-demographics and specimen characteristics of TB lymphadenitis suspects included in this study (n=144).

Socio-demographics		**N (%)**
Sex	Male	66 (45.8)
	Female	78 (54.2)
Age	1-15	21 (14.6)
	16-30	84 (58.3)
	31-45	25 (17.4)
	>45	14 (9.7)
Specimen characteristics		**N (%)**
Lymph node sites	Cervical	100 (69.4)
	Axillary	30 (20.8)
	Inguinal	14 (9.7)
Specimen appearance	Purulent	73 (50.7)
	Caseous	59 (41.0)
	Bloody stained	12 (8.3)

Culture on LJ medium was taken as a reference standard. Mycobacterial culture was found positive in 66.7% (96/144) and negative from the remaining 33.3% (48/ 144) of the TBLN presumptive cases. Among culture positives, 25% (24/96) were detected by ZN microscopy and 45.8% (44/96) by LED microscopy. Among culture negatives, 93.8% (45/48) were correctly identified as negative by ZN microscopy and 89.6% (43/48) by LED microscopy. Compared to culture, the sensitivity of ZN and LED microscopy was 25.0% and 45.8%, respectively. LED microscopy increased the sensitivity of conventional ZN microscopy by 20.8% ($P < 0.05$). The specificity was 93.8% for conventional ZN microscopy and 89.6% for LED microscopy ($P > 0.05$; **Table 2**).

Cytology was suggestive for TB in 70.1% (101/144) of total TBLN presumptive cases, of which 21.8% (22/101) were culture negatives. A total of 82.3% of culture-positive cases were classified as TB on cytological examination. Cytology has the highest sensitivity and yields few false negatives. But cytology has the lowest specificity of 54.2%. LED microscopy has greater specificity (89.6%) and yields fewer false-positive results than cytology but with lower sensitivity (45.8%).

Of 144 samples analyzed, 18.8% (27/144) were positive for acid-fast bacilli on conventional ZN microscopy; 34.0% (49/144) were positive on LED microscopy. The difference in detection yield was 17.5% (25/144). However, three cases diagnosed positive by conventional ZN microscopy were missed by LED microscopy.

Table-2: Diagnostic accuracy of conventional ZN microscopy, LED microscopy and cytology using culture as reference standard.

	ZN Microscopy	Cytology	LED microscopy
Sensitivity [95%CI]	25.0% [16.3 - 33.7]	82.3%[74.6 - 89.9]	45.8%[35.9 - 55.8]
Specificity [95%CI]	93.8% [86.9 – 100]	54.2%[40 – 68.3]	89.6%[80.9 – 98.2]
PPV [95%CI]	88.9% [77 – 100]	78.2%[70.2 – 86.3]	89.8%[81.3 - 98.3]
NPV [95%CI]	38.5% [29.6 –47.3]	60.5%[45.8 -75.1]	45.3%[35.3 – 55.3]
LR(+)[95%CI]	4 [1.3 – 12.6]	1.8 [1.3 – 2.5]	4.4 [1.9 – 10.4]
LR (-)[95% CI]	0.8 [0.7 – 0.9]	0.3 [0.2 - 0.5]	0.6 [0.5 – 0.7]

PPV=positive predictive value, NPV= negative predictive value, LR=Likelihood ratio

The mean time spent to read a positive smear was 2.65 min/slide (95% CI: 2.23–3.07) for conventional ZN method and 1.6 min/slide (95% CI: 1.34–1.86) for LED microscopy. The reading time with LED microscopy was 1.3 min for ZN smear positives (n = 24) and 1.9 min for ZN negative (n = 25). There was significantly reduced time of examination on LED microscopy compared to the conventional ZN microscopy based on non-overlapping 95% CIs. More than 93% of positive slides on LED microscopy were correctly identified within 3 min, whereas only

57.7% were by conventional ZN method. Increasing reading time from 3 to 5 min significantly increased the yield of ZN microscopy.

Based on cytomorphological diagnosis, TBLN was diagnosed in 70.1% (101/144), chronic inflammation in 10.4% (15/144), suppurative abscess in 8.3% (12/144), reactive lymphadenitis in 7.6% (11/144) and malignancy in 3.5% (5/44) of the cases. Among cases with suggestive cytomorphology of TBL, 39.6% (40/101) were diagnosed positive by LED microscopy and 22.8% (22/101) by ZN microscopy. Cytomorphological features consistent with TB were 2.5 times more likely to be positive by LED microscopy than to non-TB cytomorphological features [P-value = 0. 03, OR = 2.5, 95% CI = 1.1–5.7] (**data not indicated here).** More than 33% of the cases cytomorphologically diagnosed as non-TB suppurative abscesses were positive for AFB on LED microscopy (**Table 3**).

Table-3: Comparisons of cytomorphological diagnosis and gross specimen appearance with LED microscopy detection rate (n=144).

		LED microscopy		
		Positive	Negative	p-value
Cytomorphological diagnosis	TB lymphadenitis	39.6%(40/101)	60.4%(61/101)	
	Chronic inflammation	26.7%(4/15)	73.3%(11/15)	
	Suppurative abscess	33.3%(4/12)	66.7%(8/12)	0. 03
	Reactive LN*	9.1%(1/11)	90.0%(10/11)	
	Malignancy	0%(0/5)	100%(5/5)	
Gross FNA appearance	Purulent	32.9%(24/73)	67.1%(49/73)	
	Caseous	33.9%(20/59)	66.1%(39/59)	0. 83
	Bloody stained	41.7% (5/12)	58.3%(7/12)	

5.5. Discussion

Conventional diagnostic tools (cytology and ZN staining) for the diagnosis of EPTB have notable shortcomings due to the paucibacillary nature of the specimens and nonspecific morphological features seen in FNA cytology [10, 17]. Based on WHO's recommendation to implement LED

microscopy as an alternative to ZN microscopy for routine TB diagnosis, we were interested to study its role for the diagnosis of TBLN [13]. In agreement with previous studies [12, 20, 21], we found that LED microscopy was more sensitive than the conventional ZN microscopy for detection of AFB in lymph node aspirate. There were 25 cases which were AFB positive on fluorescent staining but were negative on conventional ZN staining. However, only three cases were positive on ZN and found to be negative on LED microscopy. LED microscopy identified a significant proportion of TBL cases missed by conventional ZN microscopy.

Lower specificity of LED microscopy compared with conventional ZN microscopy has been reported previously [21, 22]. Most studies [14, 23–25] found that the specificity of LED microscopy was comparable with that of conventional ZN microscopy. In the present study, the specificity of LED microscopy was slightly lower than that of ZN microscopy, but the difference was not statistically significant. We found that scanty AFB results on LED microscopy were less likely to be associated with a positive culture result. It is thus more likely that mycobacteria from paucibacillary specimens were killed during decontamination process and failed to grow in culture.

Examining slides with LED microscopy took significantly less time than by ZN microscopy, as reported in previous studies [14, 24]. Slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. With LED microscopy, TB bacilli stood out as bright objects against a dark background, which makes them easily identifiable hence causing less eye strain and faster grading of smears. In Ethiopia with its high rate of TB infection, technicians spend less than the recommended time to examine smears because of the high laboratory workload. LED microscopy can greatly reduce the time needed for examination of smears, thereby increasing the number of smears to be examined without compromising accuracy.

Our results showed that cytology has relatively high sensitivity but lower specificity. This is due to the fact that cytomorphological features in FNA cytology lack specificity and patients could also be diagnosed as TB. Cytology and LED microscopy offer different advantages in terms of sensitivity and specificity. Cytology yields few false negatives but has low specificity. LED microscopy has higher specificity and yields few false-positive readings.

Even though cytology is more sensitive, the cytomorphological features of suppurative abscess are not exclusive to TB. Of course, 33.3% and 26.7% of cytomorphological features of abscess and chronic inflammation of them were positive on LED test, respectively. Both methods offer different advantages in terms of sensitivity and specificity. Cytology yields few false negatives but has low specificity. The possible explanation for the misdiagnosis of specimens as suppurative abscess on cytology but positive by LED microscopy may be the absence of scattered epithelioid cells among the polymorphous population of lymphoid cells or due to the super infection of the site by other bacteria. Considering the advantage of LED microscopy among cases diagnosed as non-TBLN can improve patient management. Moreover, as cytology is less specific as shown by our current and previous study [26], it is recommended to supplement the diagnostic algorithm with LED microscopy and culture if the facility is in place. Moreover, LED microscopy can be considered as an alternative in facilities where FNA cytology is impractical.

Our study participants were recruited from a specialized referral hospital, and the specimen was processed in specialized laboratory for tuberculosis research. So, the findings of the current study may not reflect the true picture of the accuracy of the diagnostic methods in peripheral settings, which could potentially differ in terms of facilities, skilled personnel and workload. A multicenter study conducted by Cuevas et al. [27] in peripheral settings on pulmonary tuberculosis presumptive cases showed comparable sensitivity and specificity of both LED microscopy and ZN microscopy. Hence, we recommend additional studies to be conducted in real-life settings, if facilities allow diagnosing TBLN is as this is not practical at the moment in Ethiopian situation.

5.6. Conclusion

In conclusion, our study confirms that LED microscopy has higher sensitivity and comparable specificity in detection of TB bacilli in fine-needle aspirates as compared to ZN microscopy. Supplementing cytology with LED microscopy can increase the specificity and improves management of patients suspected of having TBLN. Moreover, the longer lifespan of the LED systems, the faster reading time of smears and ease of use would make this tool preferable for peripheral laboratories in resource poor settings to improve evidence-based diagnosis and treatment. Although LED shows an improvement over ZN, it remains a suboptimal diagnostic technique and that development of better diagnostic tools remains imperative.

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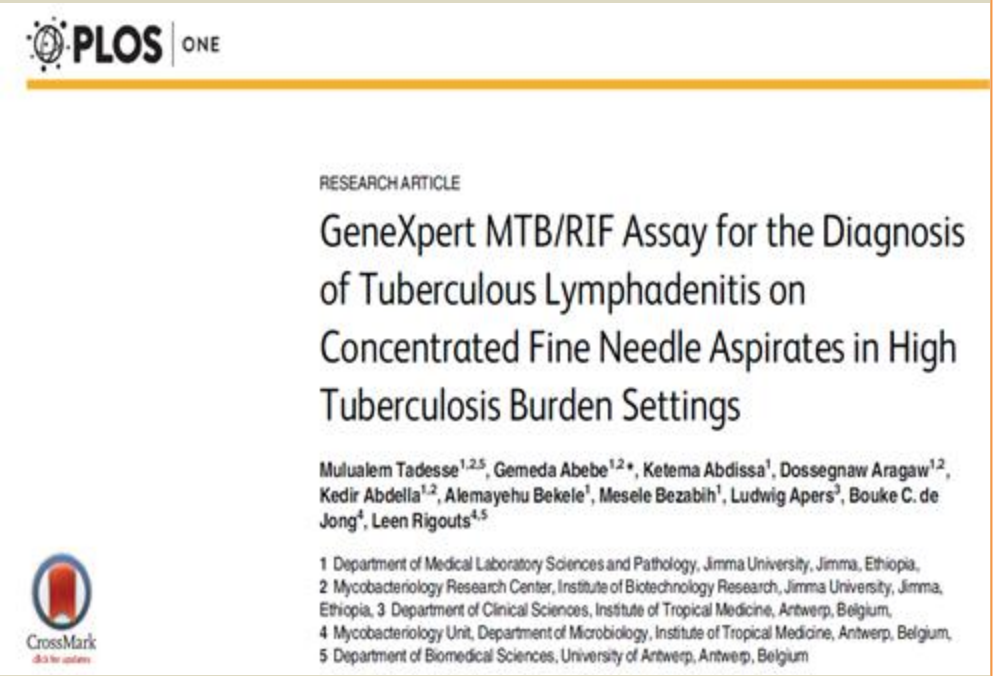
Chapter six: Paper IV

6. GeneXpert MTB/RIF assay for the diagnosis of tuberculous lymphadenitis on concentrated fine needle aspirates in high TB burden setting

This chapter is published as



PLOS ONE

RESEARCH ARTICLE

GeneXpert MTB/RIF Assay for the Diagnosis of Tuberculous Lymphadenitis on Concentrated Fine Needle Aspirates in High Tuberculosis Burden Settings

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Wrote the paper: MT, LA, BJ, LR

6.1. Abstract

Introduction: The diagnosis of tuberculous lymphadenitis (TBLN) remains challenging. The routinely used methods (cytology and smear microscopy) have sub-optimal sensitivity. Recently, WHO recommends GeneXpert to be used as the initial diagnostic test in patients suspected of having extra-pulmonary tuberculosis (EPTB). However, this was a conditional recommendation due to very low-quality evidence available and more studies are needed. In this study we evaluated the performance of Xpert MTB/RIF for the diagnosis of TBLN on concentrated fine needle aspirates (FNA) in Southwest Ethiopia.

Methods: FNA was collected from presumptive TBLN cases. Two smears were prepared from each aspirate and processed for cytology and conventional microscopy. The remaining aspirate was treated with N-acetyl-L-cysteine-NaOH and centrifuged for 15minutes at 3000g. The concentrated sediment was used for culture and Xpert MTB/RIF test. Capilia TB-Neo test was used to differentiate *M. tuberculosis* complex (MTBC) from non-tuberculous mycobacteria (NTM). Composite bacteriological methods (culture and/or smear microscopy) were considered as a reference standard.

Result: Out of 143 enrolled suspects, 64.3% (92/143) were confirmed TBLN cases by the composite reference standard (CRS). Xpert MTB/RIF detected *M. tuberculosis* complex (MTBC) in 60.1% (86/ 143) of the presumptive TBLN cases. The sensitivity of Xpert compared to CRS was 87.8% [95% CI: 81.0–94.5] and specificity 91.1% [95% CI: 82.8–99.4]. The sensitivity was 27.8% for smear microscopy and 80% for cytology compared to CRS. Cytology showed the lowest specificity (57.8%). Xpert was positive in 4 out of 45 culture- and smear-negative cases. Among 47 cytomorphologically non-TBL cases, 15 were positive on Xpert. More than half of Xpert-positive cases were in the range of very low cut-off threshold values (28<Ct<38). Resistance to rifampicin was identified in 4.7% (4/86) of Xpert-positive cases.

Conclusion: Xpert MTB/RIF showed a high sensitivity and specificity for the diagnosis of TBL on concentrated FNA samples. In addition, Xpert MTB/RIF offered rapid detection of rifampicin-resistant M. *tuberculosis* strains from lymph node aspirates.

6.2. Introduction

Tuberculosis (TB) remains a major public health problem in Ethiopia. Ethiopia ranks eighth in the list of the 22 high TB burden countries and 3rd in terms of the number of extra-pulmonary tuberculosis (EPTB); of which 80% is localized in lymph nodes [1, 2]. Accurate diagnosis and early treatment of TB has the potential to reduce morbidity and mortality associated with TB lymphadenitis (TBLN). However, the differential diagnosis of TBLN is broad and laboratory confirmation is of paramount importance to guide appropriate therapy [3, 4].

Cytology and conventional smear microscopy have been used as the initial diagnostic tools for TBL in resource poor settings [4, 5]. Fine needle aspiration cytology is a simple and rapid diagnostic technique, but with low specificity because of the presence of similar cytologic indicators in lesions other than those associated with TB [6, 7]. Conventional smear microscopy lacks sensitivity due to the paucibacillary nature of fine needle aspirates (FNA) [8]. Mycobacteriological culture and drug susceptibility testing are not always available in resource poor settings like Ethiopia [1, 9]. In line with these limitations more rapid and reliable methods are needed. In December 2010, WHO endorsed GeneXpert MTB/RIF (Cepheid, USA) for use in TB laboratories. The Xpert assay consists of a closed system that is based on real-time polymerase chain reaction (PCR). It can be used by operators with minimal technical expertise, enabling the diagnosis of TB and simultaneous detection of rifampicin resistance within 2 hours [10].

The Xpert assay has been validated and optimized for sputum samples to diagnose HIV associated TB and multidrug-resistant TB. WHO strongly recommends widespread use of Xpert for these groups of patients [11,12]. More recently a number of studies were done to evaluate this assay using non-respiratory clinical samples from patients suspected of having EPTB [9,13,14]. In 2014, WHO has recommended Xpert over the conventional tests (including conventional microscopy, culture or histopathology) for testing specific non-respiratory specimens (lymph nodes and other tissues) from patients suspected of having EPTB [15]. However, this was a conditional recommendation due to very low-quality evidence available. More studies are therefore needed particularly in settings with high EPTB prevalence. Thus, we evaluated the performance of Xpert for the diagnosis of TBLN using routinely collected FNA samples and compared it against cytology, smear microscopy and culture.

6.3. Materials and Methods

Ethical clearance was first obtained from the Jimma University ethical review board. A letter of permission to conduct the study was obtained from Jimma University Specialized Hospital clinical director office. All patients or guardians in case of children were requested for written consent prior to enrolment to the study. Any information concerning the patients was kept confidential. Laboratory results were reported back to the physicians for treatment initiation or decision as early as available.

This study was conducted at Jimma University Specialized Hospital, a public tertiary care hospital, in Southwest Ethiopia. A total of 143 consecutive outpatients clinically suspected of TBLN and referred by attending clinicians for TB testing were enrolled in this study. Participants' demographic and clinical information were collected using a pre-tested questionnaire. The FNA sample, at least 1ml, was collected by a pathologist in the pathology diagnostic unit. Gross specimen appearance (caseous, purulent, and/or blood stained) was recorded at the time of specimen collection. The first few drops of the aspirates were used for cytomorphological diagnosis. Air dried smears were stained with Wright's stain and examined by a pathologist. The cytological criteria for the diagnosis of TBL are based on the presence of the following cytomorphological appearances: epithelioid cell aggregate with or without Langerhans giant cells and necrosis, epithelioid cell aggregate without necrosis, necrosis without epithelioid cell aggregate or polymorphonucleocytes with necrosis [16]. TB treatment was initiated based on the cytomorphological diagnosis.

The remaining sample was processed for smear microscopy, culture and Xpert in the Mycobacteriology Research Center at Jimma University. Two drops from each specimen were used to make a smear for standard Ziehl-Neelsen (ZN) staining. Stained smears were examined for the presence of AFB under oil-immersion (100x) using a light microscope. All AFB smear positive slides were graded based on the IUATLD scale [17].

Mycobacterial culture was done on Löwenstein-Jensen (LJ) medium within 2 days of specimen collection. All FNA specimens were processed by the standard N-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method with a final NaOH concentration of 1% [17]. An equal volume of standard NALC-NALC/NaOH solution was added to the specimen and incubated for 15 minutes. After neutralization by phosphate buffered saline (PBS) and centrifugation (15

minutes at 3000g), the sediment was re-suspended in 1ml of sterile PBS. Finally 200µl of sediment was used to inoculate on two LJ slants. The laboratory strain, *M. tuberculosis* H37Rv (ATCC 27294), was used as a positive control. Random slants of LJ medium were inoculated with sterile distilled water in each run as negative controls. Culture positive results were confirmed for MTBC by Capilia TB-Neo test (TAUNS, Izunokuni, Japan).

Due to delay in transportation of Xpert cartridges, the remaining sediment was stored at -20°C. The median (IQR) delay before Xpert testing was 41 (30–45) days. Xpert test was performed using frozen and thawed sediment as previously described [18]. The sample reagent (1.5ml) supplied with the test was added in a 3:1 ratio to the sample sediment (0.5ml). The mixture was vortexed and incubated at room temperature for 15 minutes. Two ml of the reagent sample mix was then transferred to an Xpert cartridge using a Pasteur pipette and the cartridge was loaded onto Xpert (Cepheid, Dx System Version 4.0c) machine. Results were reported as positive or negative for *M. tuberculosis*, including a semi-quantitative scale based on the quantitative cycle threshold (Ct) value of probe A. Rifampicin resistance results were reported as susceptible, resistant or indeterminate.

Data were double entered and analyzed using the SPSS software package (version 16). Sensitivity, specificity, positive and negative predictive values with their corresponding 95% CIs were calculated using composite bacteriological methods (Culture for *M. tuberculosis* on LJ medium and/or smear microscopy using ZN method) as a reference standard. Study reporting and analysis were consistent with the standards for the reporting of diagnostic (STARD) accuracy studies checklist.

6.4. Results

A total of 143 patients with clinical presumptive TB presenting with lymphadenopathy were enrolled between May-September 2013. Out of these, 18.9% (27/143) were positive for TBLN on smear microscopy, 60.1% (86/143) on Xpert and 61.5% (88/143) on culture. On cytological examination, 67.1% (96/143) had cytomorphological features suggesting TBL. Overall, 64.3% (92/143) of tested cases were positive for TBLN 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) (Fig 1). The Xpert result was invalid for 1.4%

(2/143) of tests performed. Patients demographic and lymph node characteristics are shown in Table 1.

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-TBLN suggestive cases. When compared to CRS, smear microscopy had 27.8% sensitivity and 100% specificity whereas cytology showed sensitivity of 80.0% and specificity of 57.8%. Xpert was positive for *M. tuberculosis* in 86.4% (76/88) of culture-positive and 14.3% (7/49) of culture-negative cases. *M. tuberculosis* DNA was detected by Xpert in 3 out of 5 samples with contaminated cultures (Fig 1). Only one isolate was identified as NTM by the Capilia test and Xpert result was negative. MTBC isolates resistant to rifampicin were identified in 4.7% (4/86) of Xpert positive cases. Rifampicin resistance status for one MTBC positive sample (very low grade) was indeterminate.

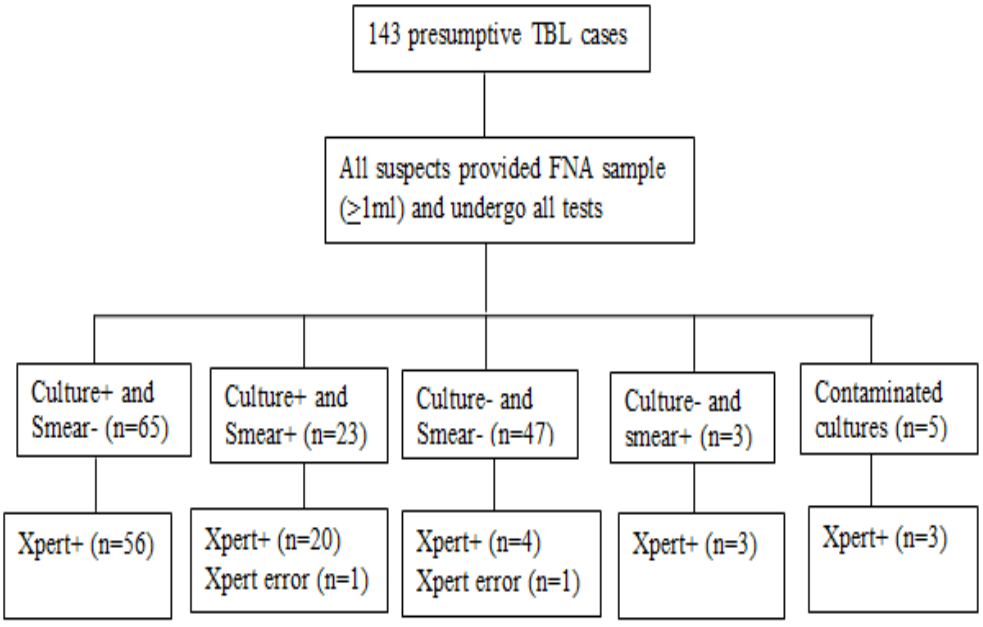

Figure-1: Flowchart for Xpert diagnostic accuracy for detection of *M. tuberculosis* from patients with suspected TBLN. TBL= tuberculous lymphadenitis

Table 1. Demographic and lymph node characteristics of TBL suspects included in this study (n=143).

Demographics characteristics		**N (%)**
Sex	Male	67 (46.9)
	Female	76 (53.1)
Age-years	≤ 15	22 (15.4)
	16-30	83 (58.0)
	31-45	23 (16.1)
	>45	15 (10.5)
Lymph node characteristics		**N (%)**
Lymph node sites	Cervical	100 (69.9)
	Axillary	29 (20.3)
	Inguinal	14 (9.8)
Specimen appearance	Purulent	73 (50.1)
	Caseous	58 (40.6)
	Bloody stained	12 (8.4)

Xpert showed an overall sensitivity of 87.8% and specificity of 91%. Xpert yielded a positive result in 56 out of 65 smear-negative/culture-positive cases. Only two specimens that were reported as “scanty AFB” in smear microscopy were negative by Xpert. When the culture positive results are stratified by AFB smear results, the sensitivity of Xpert was 91% (20/22) in smear-positive and 86.2% (56/65) in smear-negative cases. A summary of the diagnostic accuracy of cytology and Xpert test compared to CRS is presented in Table 2.

Table 2. Diagnostic accuracy of cytology and Xpert test as compared to composite reference standard (CRS).

Diagnostic accuracy	Cytology	Xpert
Sensitivity [95%CI]	80.0%[72.1 - 88.3]	87.8%[81.0 - 94.5]
Specificity [95%CI]	57.8%[43.3 - 72.2]	91.1%[82.8- 99.4]
PPV [95%CI]	79.1%[70.7 - 87.5]	95.2%[90.5- 99.8]
NPV [95%CI]	59.1%[44.6 -73.6]	78.8%[67.7- 89.9]
LR+ [95%CI]	1.9 [1.3 - 2.7]	9.8 [3.8 - 25.2]
LR- [95%CI]	0.3 [0.2 - 0.6]	0.1 [0.08 - 0.2]

PPV= positive predictive value, NPV= negative predictive value, CI=confidence interval, LR+ = likelihood ratio positive, LR- = likelihood ratio negative. The reference standard was culture for *M. tuberculosis* and/or smear microscopy for acid fast bacilli (AFB).

Xpert test assigns a semi-quantitative grade (very low, low, medium and high) on the basis of the Ct value to each test positive for *M. tuberculosis* and these categories seem to be predictive of the bacterial load [19]. Among 86 Xpert positive samples, 53.5% (46/86) were very low (28<Ct<38), 44.2% (38/86) low (22<Ct<28), and only 2 were in the 'medium' (16<Ct<22) category (Table 3). The mean Ct values were lower for smear-positive specimens compared to those smear-negative specimens (28.2 versus 31.1).

Table 3. Comparison of Xpert semi-quantitative result (Ct-value) and AFB smear grade.

	AFB smear grade				
Xpert result (Ct range)	**Negative**	**Scanty**	**1+**	**2+**	**Total (N)**
Very low (28<Ct≤38)	76% (35)	24%(11)	0	0	46
Low (22<Ct≤ 28)	71%(27)	5.3%(2)	23.7%(9)	0	38
Medium (16<Ct ≤ 22)	0	0	50.0% (1)	50.%(1)	2
High (Ct ≤ 16)	0	0	0	0	0

AFB= acid-fast bacilli; Ct= cycle threshold

The cytomorphological features consistent with TBLN were observed in 67% (96/143) of the patients with lymphadenitis. The other diagnoses reported on cytology were chronic inflammation in 11.2% (16/143), suppurative abscess in 10.5% (15/143), reactive lymphadenitis in 7.7% (11/143) and malignancy in 3.5% (5/143). Xpert detected *M. tuberculosis* in 74% (71/96) of cases with suggestive cytomorphology of TB. In addition, Xpert was also positive in 15 cases with negative cytology: 10 were from suppurative abscesses. In all these latter cases, TBLN was confirmed by the CRS (**Table 4**).

Gross lymph node aspirate was described as purulent in 51% (73/143), caseous in 40.6% (58/143) and blood stained in 8.4% (12/143) of the cases. Xpert positivity rate was highest in aspirates with caseous appearance (69% (40/58)), and lowest in blood stained aspirates (41.7% (5/12)), although these differences were statistically not significant (**Table 4**).

Table 4. Comparisons of microscopic cytomorphological features and FNA gross appearance with Xpert test and CRS (culture and/or ZN).

		Xpert	**CRS (culture and/or ZN)**
FNA cytology result	Total (N)	Positive %(n/N)	Positive %(n/N)
TBL	96	74%(71/96)	77% (74/96)
Chronic inflammation	16	18.8%(3/16)	43.8%(7/16)
Suppurative abscess	15	66.7%(10/15)	66.7%(10/15)
Reactive lymphadenitis	11	9%(1/11)	0
Malignancy	5	20%(1/5)	20%(1/5)
Gross FNA appearance			
Purulent	73	56.2%(41/73)	60.3%(44/73)
Caseous	58	69%(40/58)	72.4%(42/58)
Bloody stained	12	41.7%(5/12)	50%(6/12)

TBL= tuberculous lymphadenitis, FNA= fine needle aspirate, CRS=composite reference standard, ZN= Ziehl-Neelsen.

6.5. Discussion

The WHO and the Ethiopian national Implementation Guideline for GeneXpert strongly recommend the use of Xpert for the initial diagnosis of individuals suspected of MDR-TB or HIV associated TB [12]. Based on very low quality evidence, WHO also conditionally recommends Xpert to be used rather than conventional methods as the initial diagnostic test in patients suspected of having EPTB [12,15]. In Southwest Ethiopia, where TB and MDR-TB are highly prevalent, the effectiveness of Xpert for diagnosing TBLN and/or detection of drug resistance has not been conclusively demonstrated.

In the present study, the sensitivity of Xpert was 87.8%. A systematic review and meta-analyses conducted by Denkinger *et al* showed that Xpert test has a sensitivity ranging from 50% to 100% with pooled sensitivity of 83% [20]. More recently, Penz et al reviewed 36 studies in their meta-analyses and confirmed Xpert pooled sensitivity of 87% that is similar to our study[21]. However, the sensitivity of Xpert in the current study is lower than what was found in similar study by Ligthelm et al (sensitivity, 96.7%) [22]. There were 11 culture-positive cases which were negative on Xpert. The reason for false-negative Xpert test results may be due to the limited number of bacilli in the FNA sample or prolonged storage (median (IQR) delay of 41 (30–45) days) of sample before Xpert testing.

The specificity (91%) of the Xpert in the current study was found to be consistent with previous studies reported by others (specificity, 89–99%) [20,21,22], but higher than the study done by Biadigilegn et al (specificity, 69.2%) [13]. Seven culture-negative cases were Xpert-positive. Five of these were positive for TBLN on cytology and 3 on smear microscopy, suggesting the presence of nonviable bacilli due to either the harsh decontamination process or the nature of the caseous lesion in the lymph node tissue which may have contained dead tubercle bacilli. Such cases (Xpert-positive but culture-negative) are likely to be true TB positives as corroborated by the high specificity [14,18] and by the fact that the procedure is less prone to contamination due to the closed reaction chamber (real-time PCR technology) of Xpert.

Even though conventional ZN microscopy has played an important role in the diagnosis of TBL in resource poor settings, Xpert detected MTB in 86% (56/65) of cases missed by smear microscopy. Only 2 smear-and culture-positive samples were negative by Xpert. In agreement with other studies [9,18,22], Xpert has a higher sensitivity than smear microscopy. There was little difference in the sensitivity of Xpert in smear-positive and smear-negative TBLN cases.

Xpert detected a significant proportion of smear-negative and culture-positive cases and significantly increased the relative proportion of diagnosed TBLN cases.

In developing countries, smear microscopy is the only widely implemented method for quantifying the bacterial burden at the time of the initial diagnosis [23]. Xpert provides a semi-quantitative measurement of the number of MTBC bacilli present in a clinical sample. In this study, more than 90% of Xpert-positive samples were scored as 'low' and 'very low' suggesting a limited number of bacilli in FNA sample.

FNA cytology as an inexpensive and reliable tool for TBLN has been studied by a number of investigators [4,24,25]. It is one of the most commonly used methods in resource poor settings. In the current study the sensitivity of cytology was comparable to that of Xpert, but the specificity was lower (57.8%), yielding many false positives. This may be due to non-specific cytomorphological features seen in cytology. On the other hand, cytomorphological features associated with suppurative abscess did not reliably exclude TBLN in our study, which may be explained by the absence of characteristic features such as scattered epithelioid cells among the polymorphous population of lymphoid cells - indeed, our findings suggest that 'suppurative' features should be considered as suggestive of TB as the cause of the lymphadenitis.

To the best of our knowledge, no information regarding the drug resistance pattern of mycobacterial strains isolated from TBLN patients in Southwest Ethiopia is available. Xpert test offers rapid detection of rifampicin resistant MTBC strains directly from the clinical sample, an important advantage over cytology and smear microscopy. Previous studies reported 98–100% agreement in detection of rifampicin resistance strains using the Xpert test and phenotypic drug susceptibility test [13,14,18,26]. In the current study, rifampicin resistance was identified in 4.7% (4/86) of Xpert-positive cases. Two of these were retreatment cases.

Our study has some limitations. Mycobacterial culture on LJ medium and/or smear microscopy was used as a reference standard though both of these methods are not sufficient to detect all TBLN cases. Among culture and/or smear-negative cases there may be false negatives that started anti-TB treatment on clinical grounds and improved, cases that were most likely true TB. Unfortunately we did not include clinical outcomes in our data set. Thus, further prospective studies are required to evaluate the performance of Xpert on unprocessed fresh FNA samples by using a more sensitive liquid culture and/or histology as a reference standard

or by adding clinical diagnosis (with response to treatment) to the standards. Moreover, while we only identified one NTM in culture, we did not speciate it, and our study is unable to reflect on the contribution in Ethiopia of NTMs known to cause lymphadenitis, such as *M. scrofulaceum, M. avium* complex, and *M. kansasii*.

6.6. Conclusion

Our findings indicate that Xpert MTB/RIF test is a useful tool for the detection of MTBC with high sensitivity and specificity on concentrated fine needle aspirate with superior performance as compared to cytology and smear microscopy. Besides improved sensitivity, the Xpert was able to identify patients with TBLN due to rifampicin resistant TB. The Xpert test is an easy and suitable method to be used in TB endemic settings and its implementation could significantly improve the rapid diagnosis of TBLN.

6.7. References

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Chapter seven: Paper V

7. Clinical utility of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis in Ethiopia

This chapter discuss unpublished manuscript. The manuscript will be published as indicated below

Clinical utility of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis in Ethiopia

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7.1. Abstract

Introduction: The diagnosis of extrapulmonary tuberculosis (EPTB) is often made on clinical suspicion alone, resulting in both under- and over diagnosis and relatively poor outcomes. In this study, we evaluated the clinical utility of the Xpert MTB/RIF on routinely collected extra-pulmonary specimens in Ethiopia.

Methods: This study was carried out at Jimma University Specialized Hospital, Southwest Ethiopia, from September 2015 to June 2017. Extra-pulmonary specimens were collected from 572 patients clinically suspected of suffering from EPTB. All specimens were tested for TB by smear-microscopy, culture and Xpert MTB/RIF. The diagnostic accuracy of Xpert MTB/RIF was calculated compared to a composite reference standard (CRS), comprising clinical and laboratory results.

Results: In total, 572 extra-pulmonary specimens (279 lymph node, 159 pleural, 80 peritoneal, 45 cerebrospinal and 9 pericardial fluids) were tested. The pooled sensitivity and specificity of Xpert MTB/RIF were calculated to be 75% and 98% respectively when compared to CRS. The highest sensitivity was documented for lymph node specimen (90%), moderate sensitivity for cerebrospinal fluid (53%), while the sensitivity was lowest for pleural (30%) and peritoneal (32%) fluids. Xpert MTB/RIF in addition detected rifampicin resistance in 13 patients, in perfect agreement with results from line probe assay.

Conclusions: Xpert MTB/RIF may be used as initial diagnostic tool for testing of lymph node specimens from patients suspected of having TB lymphadenitis. The added value of Xpert MTB/RIF to diagnose pleural or peritoneal TB is limited by its poor sensitivity.

Keywords: Extrapulmonary tuberculosis, Xpert MTB/RIF, diagnostic accuracy, Ethiopia

7.2. Introduction

Tuberculosis (TB) that affects any organ outside the pulmonary parenchyma is designated as extrapulmonary tuberculosis (EPTB). EPTB is a significant public health problem worldwide [1], and can potentially affect any organ in the body, with some preferred sites: superficial and deep lymph nodes, pleura, bones and joints, the central nervous system (CNS), the abdomen (gastrointestinal, peritoneum) and genitourinary tracts. A small number of cases present with disseminated TB [2]. Ethiopia, a country that ranks 10th among high TB burden countries, has an extremely high rate of EPTB, dominated by lymph node TB [3]. The proportion of EPTB was reported to be as high as 40% across Ethiopia compared to 15-20% worldwide [3, 4]. The diagnosis of EPTB is challenging for several reasons. The variable non-specific presentations, paucibacillary nature of the disease, non-uniform distribution of bacilli, difficulty in obtaining appropriate and adequate samples, and poor performance of conventional microbiological techniques in EPTB all contribute to challenges in diagnosing EPTB [2, 4, 5]. This problem particularly affects resource-limited settings, where the more sensitive methods of mycobacterial culture and histological examination are not widely available. These all lead to delayed or missed diagnosis with increased morbidity and mortality, or over diagnosis leading to unnecessary TB treatment.

Among many nucleic-acid amplification tests, the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is well-established technology that has been endorsed by WHO for the initial diagnosis of individuals suspected of rifampicin-resistant or HIV-associated TB, and as a follow-on test to microscopy in smear-negative specimens [6, 7]. More recently, assessments of the Xpert MTB/RIF have extended to various non-respiratory clinical samples from patients with EPTB [8-11], showing highly heterogeneous sensitivity between specimen types, ranging from 25% to 96.6%. In 2013, WHO updated a new guideline, recommending the use of Xpert MTB/RIF for some types of EPTB such as TB lymphadenitis and TB meningitis [12].

Xpert MTB/RIF has been nationally implemented in Ethiopia in selected laboratories as a replacement for smear microscopy for initial diagnosis of TB and screening of drug resistant TB [13]. The ministry of health has a plan to scale-up the implementation of Xpert MTB/RIF throughout the laboratories. In our previous study [14], we reported excellent sensitivity (87.8%) and specificity (91%) of Xpert MTB/RIF for the diagnosis of TB lymphadenitis using processed fine needle aspirate. However, in that study, patients' clinical data sets were not

considered and Xpert MTB/RIF has been compared to Löwenstein-Jensen (L-J culture) and/or smear microscopy, which is known to be a suboptimal reference standard for EPTB. Therefore, we carried out the current study to investigate the use of Xpert MTB/RIF for diagnosing EPTB on different types of extra-pulmonary specimens routinely received at Jimma University Specialized Hospital by using a composite reference standard (CRS), composed of a more sensitive liquid culture (MGIT 960), smear-microscopy, cytological and radiological findings and treatment response.

7.3. Materials and Methods

7.3.1. Study population and specimen

This prospective hospital-based study was conducted at Jimma University Specialized Hospital, a public tertiary care hospital, Southwest Ethiopia. Consecutive patients (age $\geq$ 15 years) with signs and symptoms suggestive of EPTB were included in the study from September 2015 to June 2017. Site specific extra-pulmonary specimens were collected from patients with presumed EPTB and sent to the Mycobacteriology Research Center for laboratory diagnosis. The collected specimens were processed for fluorescence microscopy, mycobacterial culture (BACTEC 960 MGIT) and Xpert MTB/RIF. Personnel involved in performing and reporting of the Xpert MTB/RIF were blinded to the results of microscopy and culture. Patient's medical records were examined for a clinical diagnosis of TB: radiology findings, cytology reports and/or clinical improvement after anti-TB treatment.

7.3.2. Laboratory processing of specimens

All microbiological investigations were carried out at Jimma University-Mycobacteriology research Center (JU-MRC). Upon receipt in the JU-MRC, the specimen was divided in two parts: the first part was used for Xpert MTB/RIF and the second for culture and smear-microscopy. For FNA (usually 0.5-1mL volume), the volume was raised to 2 mL by addition of phosphate buffered saline (PBS) and split in two prior to testing.

MGIT 960: Culture was performed using liquid medium (MGIT 960; Becton Dickinson Biosciences, Sparks, MD, USA). A different pre-treatment was adopted according to the specimen type. Non-sterile specimens as well as bloody stained specimens were decontaminated by adding an equal volume of N-acetyl –L-cysteine sodium hydroxide –NaOH (1% final concentration) and incubating for 15 minutes at room temperature. After centrifugation for 15 minutes at 3000g, the sediment was re-suspended in 1mL of sterile

phosphate buffered saline [15]. Specimens expected to be sterile were directly centrifuged to concentrate the samples. MGIT tubes were inoculated with 0.5 mL of the processed specimen and incubated in the MGIT 960 instrument at 37°C. For tubes identified as positive, a smear was prepared to detect acid-fast bacilli (AFB). Culture-positive results were confirmed for MTBc by a *p*-nitro-benzoic acid test [15].

AFB-smear microscopy: Smears were prepared from concentrated sample sediments except for FNA, which was smeared unprocessed. All smears were stained by auramine O and examined by light-emitting diode-fluorescence microscopy for the presence of AFB. Observation of a single AFB was considered a positive result.

Xpert MTB/RIF: The Xpert MTB/RIF assay was performed as previously described [16]. Specimens were processed directly by the addition of a 2:1 volume of sample reagent. The mixture was vortexed and incubated at room temperature for 15 minutes. Two mL of the reagent-sample mix was then transferred to an Xpert cartridge using a Pasteur pipette and the cartridge was loaded onto GeneXpert (Cepheid, Dx System Version 4.0c) machine. If rifampicin-resistance was detected by the Xpert MTB/RIF, further drug susceptibility testing by the GenoType MTBDR*plus* line probe assay was performed on DNA extracted from a positive MGIT culture as per the manufacturer's instruction (Hain Life science, Nehren, Germany) [17].

7.3.3. Diagnostic classification for analysis

Based on the results of smear microscopy, culture, radiology, cytology and clinical response after ATT, study participants were categorized into the following diagnostic groups: (i) Confirmed TB: defined as a positive culture of MTBc regardless of smear result; (ii) Probable TB: patients not meeting the criteria for confirmed-TB but TB was suggested with the fulfillment of one of the following criteria: positive smear microscopy, cytological or radiological features suggestive of TB, or clinical improvement after ATT; (iii) Non TB: patients for whom no microbiological (smear-negative and culture-negative) or cytological evidence of TB could be found, and/or for whom an alternative diagnosis was available (none of the patients in this category received ATT); (iv) Indeterminate: patients not meeting the confirmed and probable TB criteria and/or patient medical records were lost or incomplete and clinical diagnosis of TB was not made. For patients in this category (indeterminate), the diagnoses of TB remained uncertain and were excluded from analysis against CRS (**Figure 1**).

7.3.4. Statistical analysis

Sensitivity, specificity, positive (PPV) and negative (NPV) predictive values with 95% CIs were calculated for Xpert MTB/RIF using culture positivity from the same specimen as a reference standard. These calculations were performed on the total sample size as well as for individual specimen types. Xpert MTB/RIF diagnostic accuracy was also assessed in comparison to a composite reference standard (CRS) made up of smear and culture results, radiological and cytological findings and clinical improvement after ATT. Any patient that was positive for any one component of the CRS was considered 'TB'. Overlapping CI data were regarded as showing no significant difference between the results determined for the corresponding sample types. The Xpert MTB/RIF invalid rate was calculated by dividing the number of specimens classified as "invalid or error" to the total number of specimens analyzed by Xpert MTB/RIF. The culture contamination rate was calculated by dividing the number of specimens contaminated in culture to the total number of specimens cultured.

7.3.5. Ethical approval

Ethical approval for this study was obtained from the Ethical Review Board of Jimma University, Ethiopia (Ref. No. RPGC/510/2014) and the Institutional Review Board of Institute of Tropical Medicine, Antwerp, Belgium (Ref. No. 986/15). Written informed consent was obtained from each patient. For study participants who could not read and write, an impartial witness was co-signed. Laboratory results were reported back to the physicians for treatment initiation or decision as early as available.

7.4. Results

A total of 585 patients referred to Jimma University Specialized Hospital with presumed EPTB were eligible. Of these, 13 patients provided insufficient specimen volume and excluded from the study. In the remaining 572 patients, site specific extra-pulmonary specimens were obtained (one specimen per patient). These comprised 279 lymph node specimens, 45 cerebrospinal fluid (CSF), 159 pleural, 80 peritoneal and 9 pericardial fluids. All specimens were processed for fluorescence smear-microscopy, culture and Xpert MTB/RIF. Twenty seven specimens with contaminated cultures and 3 samples that grew NTM were removed from analysis against culture as the reference standard. In addition to microbiological results, clinical details, cytological and/or radiological findings, and treatment responses were reviewed from patient medical records and patients were categorized to different diagnostic

groups. Of 572 patients, 226 (39.5%) were culture-positive "confirmed TB" cases, 83 (14.5%) "probable TB" cases were clinically, radiologically and/or cytologically positive and received ATT with good response, and 155 (27%) were classified as "non TB" cases because of no evidence for TB. In the remaining 108 (19%) patients, TB diagnosis was uncertain "indeterminate cases" (**Figure 1**).

7.4.1. Clinical and demographic data

Of 572 patients, 295 (51.6%) were females. The mean age of the patients was 33.3 (± 12SD) years. HIV test results were available for 449 patients and 64 of the patients (14.3%) were positive for HIV. Overall, 226 (39.5%) of the 572 specimens were positive for MTBc by culture. Out of 225 positive cultures, 106 (47%) were also smear positive for AFB. The majority (n=197) of MTBc strains were isolated from lymph node specimens, and the remaining 13, 8, 7 and 1 from pleural, CSF, peritoneal and pericardial specimens respectively. Demographic and clinical data are summarized in **Table 1**.

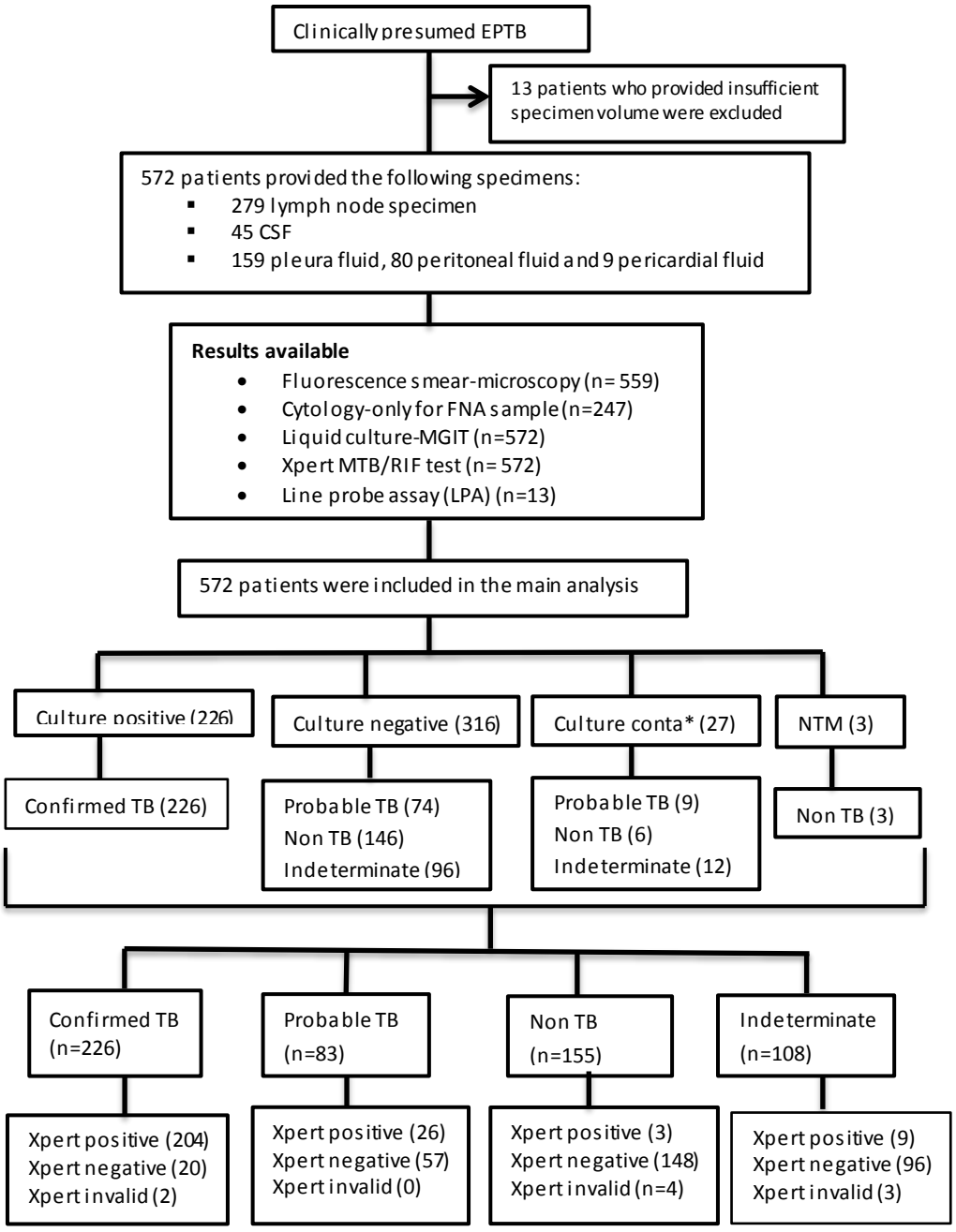

Figure 1: Flowchart explaining the overall patient flow and diagnostic classifications. CSF= cerebrospinal fluid, CRS= composite reference standard, NTM= non-tuberculous mycobacteria, *contamination. Indeterminate cases i.e. patients with uncertain diagnosis were excluded from CRS reference standard.

Table 1: Demographic and clinical characteristics of patients referred to Jimma University Specialized Hospital with presumed EPTB.

Characteristics	All patients (N=572)	Confirmed TB (n=226)	Probable TB (n=83)	Non TB (n=155)	Indeterminate (n=108)
Age					
15-25	205 (35.8)	93 (41.2)	28 (33.7)	47 (30.3)	37 (34.3)
26-35	179 (31.3)	71(31.4)	28 (33.7)	46 (29.7)	34 (31.5)
36-45	92 (16.1)	32 (14.2)	11 (13.3)	2 9(18.7)	20 (18.5)
46-55	57 (10.0)	23 (10.2)	8 (9.6)	16 (10.3)	10 (9.3)
≥56	39 (6.8)	7 (3.1)	8 (9.6)	17 (11.0)	7 (6.5)
Gender					
Male	277 (48.4)	100 (44.2)	39 (47)	90 (58.1)	48 (44.4)
Female	295 (51.6)	126 (55.8)	44(53)	65 (41.9)	60 (55.6)
HIV status					
Positive	64 (11.2)	20 (8.8)	8 (9.6)	21 (13.5)	15 (13.9)
Negative	385 (67.3)	176 (77.9)	63 (75.9)	91 (58.7)	55 (50.9)
Unknown	123 (21.5)	30 (13.3)	12 (14.5)	43 (27.7)	38 (35.2)
Specimen type					
Lymph node	279 (48.8)	197 (87.2)	30 (36.1)	38 (24.5)	14 (13.0)
CSF	45 (7.9)	8 (3.5)	7 (8.4)	21 (13.5)	9 (8.3)
Pleural	159 (27.8)	13 (5.8)	30 (36.1)	64 (41.3)	52 (48.1)
Peritoneal	80 (14.0)	7 (3.1)	15 (18.1)	30 (19.4)	28 (25.9)
Pericardial	9 (1.6)	1 (0.4)	1 (1.2)	2 (1.3)	5 (4.6)
Smear-result**					
Positive	117 (20.9)	106 (47.1)	10 (12.3)	0	0
Negative	442 (79.1)	119 (52.9)	71 (87.7)	147 (100)	106 (100)

CSF= cerebrospinal fluid, **smear-result for 13 patients was not available.

7.4.2. Performance of Xpert MTB/RIF compared to MGIT culture

MTBc was detected in 42.3% (242) of specimens by Xpert MTB/RIF and in 39.5% (226) by MGIT culture. The Xpert MTB/RIF and culture positivity rate was highest in lymph nodes (75.6% and 70.6% respectively), modest in CSF (17.8% each by Xpert MTB/RIF and culture) and lowest in other fluids (9.3% and 8.5% respectively). The Xpert MTB/RIF result was invalid for 1.6% (9) of tests performed, a rate lower than the overall contamination rate for MGIT culture which was

4.7% (27) (**Table 2**). In 9 of 27 culture contaminated cases, MTBc was detected by Xpert MTB/RIF and in all 9 cases, TB was clinically diagnosed (**Table 3**). Xpert MTB/RIF gave positive results in 29 (9.2%) of 316 culture negative patients; for 17 of these patients, TB was clinically confirmed while for three cases an alternative diagnosis was made and TB was ruled-out. In the remaining nine, no detailed clinical data were obtained and no confident diagnosis of TB could be made. Therefore, they were only included in the analysis comparing Xpert MTB/RIF to culture results.

Table 2: MTBc detection rate of Xpert MTB/RIF and culture with respect to the different types of specimens.

Specimen type	Xpert MTB/RIF			MGIT culture			
	Positive n (%)	Negative n (%)	Invalid n (%)	Positive n (%)	Negative n (%)	Contam n (%)	NTM n (%)
Lymph node (279)	211 (75.6)	65 (23.3)	3 (1.1)	197 (70.6)	63 (22.6)	16 (5.7)	3 (1)
CSF (n=45)	8 (17.8)	36 (80.0)	1 (2.2)	8 (17.8)	37 (82.2)	0	0
Pleural (n=159)	14 (8.8)	142 (89.3)	3 (1.9)	13 (8.2)	143 (89.9)	3 (1.9)	0
Peritoneal (n=80)	9 (11.2)	69 (86.2)	2 (2.5)	7 (8.8)	66 (82.5)	7 (8.8)	0
Pericardial (n=9)	0	9 (100)	0	1 (11.1)	7 (77.8)	1 (11.1)	0
All samples (n=572)	242(42.3)	321(56.1)	9 (1.6)	226(39.5)	316(55.2)	27(4.7)	3 (0.5)

CSF= cerebrospinal fluid, NTM= non-tuberculous mycobacteria, MGIT= mycobacteria growth indicator tube

Overall, the pooled sensitivity and specificity of Xpert MTB/RIF were calculated to be 91% [95% CI: 87.3 - 94.8] and 90.6% [87.4 - 93.8], respectively when compared to culture (**Table 4**). The sensitivity of Xpert MTB/RIF (91%) was significantly higher than smear microscopy performed on the same specimen, which had a sensitivity of 47% [95% CI: 40.6-53.6] (data not shown). The PPV and NPV of Xpert MTB/RIF were 87.6% [95% CI: 83.3-91.8] and 93.4% [95% CI: 90.5 - 96.2], respectively. The Xpert MTB/RIF sensitivity differed markedly between the specimen types. While the Xpert MTB/RIF detected 184 (93.4%) out of 197 culture-positive cases among lymph node specimens, the Xpert MTB/RIF detected only 14 (66.7%) out of 21 culture-positive cases among other specimens (**Table 3**). The analysis of Xpert MTB/RIF diagnostic performance by specimen type was presented in **Table 4**. Xpert MTB/RIF had the highest sensitivity on

lymph node specimen (94.6% [95% CI: 91-98]) followed by CSF (75% [95% CI: 45-100]). The Xpert MTB/RIF sensitivity was lowest for pleural (69% [95% CI: 44-94]) and peritoneal fluids (71% [95% CI: 38-98%]), with non-overlapping CIs between lymph node and pleural or peritoneal fluids.

The Xpert MTB/RIF specificities varied to a lesser extent across the different specimen types, with fluid specimens (pleural, peritoneal and CSF) showing the highest specificity ($\geq$ 94%). The specificity value for Xpert MTB/RIF was lowest for lymph node specimen (71.5% [95% CI: 60-83]) (**Table 4**).

Table 3: Direct comparison of Xpert MTB/RIF with culture in different specimen types.

			MGIT culture			
Specimen type			**Positive n (%)**	**Negative n (%)**	**Contaminated n (%)**	**NTM n (%)**
Lymph node	Xpert	Positive	184 (87.2)	18 (8.5)	9 (4.3)	0
(n=279)		Negative	11 (16.9)	45 (69.2)	6 (9.2%)	3 (4.6)
		Invalid	2 (66.7)	0	1 (33.3)	0
CSF (n=45)	Xpert	Positive	6 (75.0)	2 (25.0)	0	0
		Negative	2 (5.6)	34 (94.4)	0	0
		Invalid	0	1 (100)	0	0
Pleural	Xpert	Positive	9 (64.3)	5 (35.7)	0	0
(n=159)		Negative	4 (2.8)	135 (95.1)	3 (2.1)	0
		Invalid	0	3 (100)	0	0
Peritoneal	Xpert	Positive	5 (55.6)	4 (44.4)	0	0
(n=80)		Negative	2 (2.9)	60 (87.0)	7 (10.1)	0
		Invalid	0	2 (100)	0	0
Pericardial	Xpert	Positive	0	0	0	0
(n=9)		Negative	1 (11.1)	7 (77.8)	1 (11.1)	0
		Invalid	0	0	0	0
All specimens (n=572)	Xpert	Positive	204 (84.3)	29 (12.0)	9 (3.7)	0
		Negative	20 (6.2)	281 (87.5)	17 (5.3)	3 (0.9)
		Invalid	2 (22.2)	6 (66.7)	1 (11.1)	0

CSF= cerebrospinal fluid, NTM= non-tuberculous mycobacteria, MGIT= mycobacteria growth indicator tube

Table 4: Performance of Xpert MTB/RIF test with respect to different specimen types compared to culture and CRS.

	Culture as a reference standard		CRS as a reference standard	
Specimen type	Sensitivity % (95%CI)	Specificity % (95%CI)	Sensitivity % (95%CI)	Specificity % (95%CI)
Lymph node	94.4[91-98]	71.5[60-83]	89.8[86-94]	92[84-100]
CSF	75[45-100]	94[87-100]	53[28-79]	100
Pleural	69[44-94]	96.5[93-99]	30[17-44]	100
Peritoneal	71[38-98]	94[88-100]	32[12-51]	100
Pooled	91[87-95]	90.6[87-94]	75[70-80]	98[96-100]

CSF= cerebrospinal fluid, CRS= composite reference standard, CI= confidence interval. Calculation of sensitivity and specificity for pericardial fluid was not possible due to small number of this specimen in our study.

7.4.3. Performance of Xpert MTB/RIF compared to CRS

Xpert MTB/RIF identified 90.3% (204/226 specimens) of all "confirmed TB" cases - including 101 smear-negative TB cases and 31.3% (26/83) of "probable TB" cases (**Table 5**). Using the CRS as comparator, the pooled sensitivity of Xpert MTB/RIF significantly decreased (91% vs 75% with non-over lapping CIs), except for lymph node specimen yielding a sensitivity of 90% [95% CI: 86-94]. The sensitivity was modest for CSF (53% [95% CI: 28–78]) and lowest for pleural (30% [95% CI: 17–44]) and peritoneal (32% [95% CI: 12–51]) specimens (**Table 4**).

The Xpert MTB/RIF specificity was excellent for all types of specimens tested, and ranged from 92% to 100%. Overall, the Xpert MTB/RIF specificity was 90.6% [95% CI: 87-94]) when compared to culture and improved to 98% [95% CI: 96-100] when compared to CRS (**Table 4).**

Table 5: Comparison of Xpert MTB/RIF results with CRS in different specimen types.

Specimen type			CRS			
			Confirmed-TB n (%)	Probable-TB n (%)	Non-TB n (%)	Indeterminate n (%)
Lymph node (n=279)	Xpert MTB/RIF	Positive	184 (87.2)	18 (8.5)	3 (1.4)	6 (2.8)
		Negative	11 (16.9)	12 (18.5)	35 (53.8)	7 (10.8)
		Invalid	2 (66.7)	0	0	1 (33.3)
CSF (n=45)	Xpert MTB/RIF	Positive	6 (75.0)	2 (25.0)	0	0
		Negative	2 (5.6)	5 (13.9)	20 (55.6)	9 (25.0)
		Invalid	0	0	1 (100)	0
Pleural (n=159)	Xpert MTB/RIF	Positive	9 (64.3)	4 (28.6)	0	1 (7.1)
		Negative	4 (2.8)	26 (18.3)	62 (43.7)	50 (35.2)
		Invalid	0	0	2 (66.7)	1 (33.3)
Peritoneal (n=80)	Xpert MTB/RIF	Positive	5 (55.6)	2 (22.2)	0	2 (22.2)
		Negative	2 (2.9)	13 (18.8)	29 (42.0)	25 (36.2)
		Invalid	0	0	1 (50.0)	1 (50.0
Pericardial (n=9)	Xpert MTB/RIF	Positive	0	0	0	0
		Negative	1 (11.1)	1 (11.1)	2 (22.2)	5 (55.6)
		Invalid	0	0	0	0
All specimens (n=572)	Xpert MTB/RIF	Positive	204 (84.3)	26 (10.7)	3 (1.2)	9 (3.7)
		Negative	20 (6.2)	57 (17.8)	148 (46)	96 (29.9)
		Invalid	2 (22.2)	0	4 (44.4)	3 (33.3)

CSF= cerebrospinal fluid, CRS= composite reference standard

7.4.4. Detection of rifampicin resistance

Xpert MTB/RIF testing for rifampicin resistance showed an "invalid" result in two cases. After a single repeat test, both cases were found to be rifampicin sensitive. In total, rifampicin resistance was detected in 16 patients by Xpert MTB/RIF. Of these, 2 patients were culture negative and one was contaminated, and thus line probe assay result was not available for these three cases. In the remaining 13 Xpert MTB/RIF-rifampicin resistant cases, LPA results were in full agreement. Xpert MTB/RIF-resistant cases were also found to be isoniazid resistant, making rifampicin resistance a good predictor of MDR-TB.

7.5. Discussion

WHO has strongly recommended Xpert MTB/RIF for the initial diagnosis of individuals with presumed MDR-TB or HIV-associated TB [6]. In 2013, WHO updated its endorsement and Xpert MTB/RIF has recently been conditionally recommended for some types of EPTB such as TB lymphadenitis and TB meningitis [12]. In Ethiopia, Xpert MTB/RIF is undergoing phased implementation for routine diagnosis of TB and drug resistant TB [13]. However, there are no comprehensive data on the diagnostic utility of Xpert MTB/RIF on EPTB cases to guide clinical practice in Ethiopia. Most previous studies that evaluated Xpert MTB/RIF performance have compared Xpert MTB/RIF to culture, which is known to be a suboptimal reference standard for EPTB. Therefore, we have compared Xpert MTB/RIF to culture and a CRS to evaluate the better diagnostic potential of the Xpert MTB/RIF on the different types of extra-pulmonary specimens routinely received in a tertiary health care hospital in Ethiopia.

Our study shows that Xpert MTB/RIF has a pooled sensitivity of 91% compared to culture which was reduced to 75% when compared to CRS, whereas the specificity improved from 91% to 98% when compared to culture and CRS respectively. Although culture is considered the best reference standard for pulmonary TB, it may miss cases of EPTB due to the paucibacillary nature of the disease. Thus, when Xpert MTB/RIF is evaluated against culture alone, the number of false-positive EPTB cases (classified as positive by the Xpert MTB/RIF and negative by the culture) may be overestimated, leading to an underestimation of the Xpert MTB/RIF's specificity. The use of a CRS reclassifies these to true positives and increases the specificity. The other drawback of using culture alone as a reference standard is that both culture and Xpert MTB/RIF are likely to pick up cases with a higher bacterial load, and both are likely to miss cases with a lower bacterial load (false-negative by both Xpert MTB/RIF and culture). This dependency could lead to an overestimation of the Xpert MTB/RIF sensitivity, which could be corrected for by bringing in clinical and treatment outcome data.

Our study confirms previous observations on variability in sensitivity of Xpert MTB/RIF across different specimen types, with the highest sensitivity for TB detection in lymph node specimens, moderate sensitivity in CSF, but lower sensitivity in fluid specimens such as pleural and peritoneal [12, 18-20]. For lymph node specimens, the sensitivity of Xpert MTB/RIF compared to culture or the CRS was not statistically different. More than 88% of TB lymphadenitis cases defined by the CRS were confirmed by Xpert MTB/RIF. Hence, a negative

Xpert MTB/RIF result in lymph node specimens should guide for an alternative diagnosis. This is likely because the bacillary load in lymph node specimen sampled directly from the site of disease (lymph node) is above the limit of detection of the Xpert MTB/RIF. Besides, lymph node specimens are relatively easy to obtain, with one milliliter being sufficient for accurate performance of the Xpert MTB/RIF.

For CSF, the sensitivity of Xpert MTB/RIF was modest (75%) compared to culture and reduced to 53% compared to the CRS. Hence, even though a negative Xpert MTB/RIF does not rule-out TB meningitis, it could still significantly speed up and improve its diagnosis in settings where liquid culture or better tools are not available. Although not attempted in our current study, a South African study, showed a significant increase in the sensitivity of Xpert MTB/RIF using a CSF pellet after centrifugation [21].

Similar to most previous reports [8, 18, 19], we found a low sensitivity and high specificity of Xpert MTB/RIF on fluids, whether compared to culture or the CRS. The poor sensitivity in such fluids is probably due to the paucibacillary nature of the disease. Some studies suggested tissue biopsies rather than fluids as the samples of choice for the diagnosis of paucibacillary TB [8, 18], even though the more invasive nature of the former restricts its widespread use. In our study, except for lymph node samples, the sensitivity of Xpert MTB/RIF significantly reduced when compared against the CRS, with only around 15% of probable TB cases being detected in fluid specimens. Hence, a negative Xpert MTB/RIF in pleural or peritoneal fluid does not exclude a diagnosis of EPTB. Patients whose symptoms and signs strongly suggest disseminated EPTB should be started ATT, despite a negative Xpert MTB/RIF. Clinical diagnosis remains an essential part of the diagnostic pathway until better and more sensitive tools are available. More recently, the WHO expert evaluation concluded that the new Xpert Ultra test showed better performance than Xpert MTB/RIF in detecting TB in difficult-to-diagnose and vulnerable populations, such as children and people living with HIV, and in those with extra-pulmonary TB. In high TB prevalent settings like Ethiopia, the implementation of Xpert Ultra has a potential to overcome the sensitivity limitations of Xpert MTB/RIF in pleural or peritoneal specimens [22].

Nevertheless, the high pooled specificity highlights the utility of Xpert MTB/RIF as a rule-in test for EPTB diagnosis, providing sufficient confidence for the clinician to initiate ATT following a positive Xpert MTB/RIF result. Unlike its sensitivity, the Xpert MTB/RIF specificity varied less

across different specimen types. Compared to culture, the specificity of the Xpert MTB/RIF was low for lymph node specimens (72%), is most likely due to underperformance of culture, i.e. nonviable growth of MTBc bacilli from these specimen types, while DNA amplification was not hampered. In 29 cases, the Xpert MTB/RIF result was positive but the culture remained negative. Of these, 17 patients had either radiologically or cytologically proven TB or a clinical response when treated with ATT, and no obvious reason was found to explain the negative culture. In 9 patients, no clinical data were available to resolve the observed discrepancy. For the remaining 3 cases, TB was rule-out (CRS-negative), though administrative errors or sample switch could have contributed for this discrepancy. Nevertheless, taking into account that Xpert MTB/RIF is less prone to cross contamination, being a 'closed' test system (single cartridge, real-time PCR technology), these Xpert-positive but culture- or CRS-negative cases are likely to represent true-positive cases.

Apart from providing bacteriological confirmation of disease, rapid detection of rifampicin resistance in extra-pulmonary specimens is another added benefit of Xpert MTB/RIF. In our study, LPA susceptibility testing confirmed the 13 cases of rifampicin resistance revealed by Xpert MTB/RIF. WHO has recommended that all rifampicin resistant cases should start second line TB regimens. Therefore, a rifampicin-resistant EPTB case diagnosed by Xpert MTB/RIF should be evaluated in the context of the clinical information and response to treatment and, wherever possible, should be referred for second line-LPA and/or phenotypic susceptibility testing.

Our study has some shortcomings. The sample size for certain specimen types such as CSF and pericardial fluid was limited, urging for caution when interpreting results for each category of specimens separately. Also in a significant number of patients, their medical records were lost or incomplete and the TB diagnosis remained uncertain. These cases were excluded from the CRS analysis. Finally, we believe that even though the use of CRS may introduce a minor degree of selection bias and may be difficult to standardize, this consideration outweighs the risk of misclassification when using culture alone as a reference.

7.6. Conclusions

Xpert MTB/RIF is likely to be of greatest utility when testing lymph node specimens. While Xpert MTB/RIF has modest sensitivity in CSF, it could be considered to improve the diagnosis of TB meningitis in places where culture or more sensitive tests are not available. A negative

Xpert MTB/RIF on fluid specimens such as pleural and peritoneal does not exclude the diagnosis of EPTB. Patients with a high clinical probability of EPTB despite a negative Xpert MTB/RIF should be started on anti-TB treatment until better and more sensitive tools are available. Coupled with speed and simplicity, and its ability to detect rifampicin resistance, Xpert MTB/RIF is a useful diagnostic tool that can be integrated into a routine diagnostic protocol in low income countries. However, there is still a need to evaluate and confirm the utility of Xpert MTB/RIF on a large sample size with specimens such as CSF, other body fluids, and urine, which are easier to obtain. Studies must also assess the impact of the use of Xpert MTB/RIF on time to TB diagnosis and clinical outcomes, thereby permitting cost–effectiveness analyses to be performed.

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Chapter eight: Paper VI

8. The predominance of Ethiopian specific *M. tuberculosis* families and minimal contribution of *M. bovis* in tuberculous lymphadenitis patients in Southwest Ethiopia

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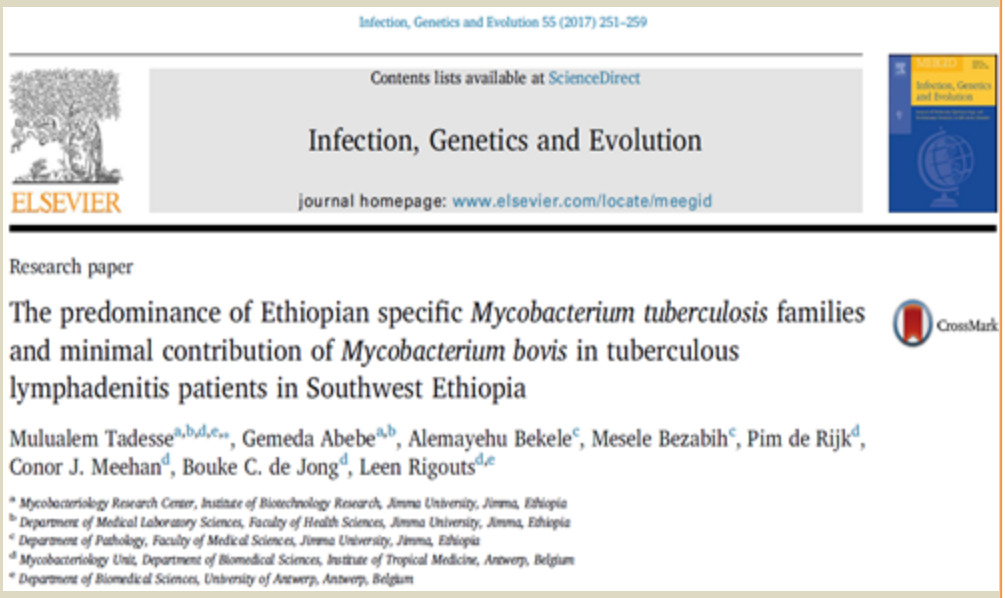

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Research paper

The predominance of Ethiopian specific *Mycobacterium tuberculosis* families and minimal contribution of *Mycobacterium bovis* in tuberculous lymphadenitis patients in Southwest Ethiopia

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Contributed reagents and materials: LR, BDJ

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8.1. Abstract

Background: Ethiopia has an extremely high rate of extrapulmonary tuberculosis, dominated by tuberculous lymphadenitis (TBLN). However, little is known about *Mycobacterium tuberculosis complex* (MTBc) lineages responsible for TBLN in Southwest Ethiopia.

Methods: A total of 304 MTBc isolates from TBLN patients in Southwest Ethiopia were genotyped primarily by spoligotyping. Isolates of selected spoligotypes were further analyzed by 15-loci mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) (n=167) and qPCR-based single nucleotide polymorphism (n=38). Isolates were classified into main phylogenetic lineages and families by using the reference strain collections and identification tools available at *MIRU-VNTRplus* data base. Resistance to rifampicin was determined by Xpert MTB/RIF.

Results: The majority of isolates (248; 81.6%) belonged to the Euro-American lineage (Lineage 4), with the ill-defined T and Haarlem as largest families comprising 116 (38.2%) and 43 (14.1%) isolates respectively. Of the T family, 108 isolates were classified as being part of the newly described Ethiopian families, namely Ethiopia_2 (n=44), Ethiopia_3 (n=34) and Ethiopia_H_{37}Rv-like (n=30). Other sub-lineages included URAL (n=18), S (n=17), Uganda I (n=16), LAM (n=13), X (n=5), TUR (n=5), Uganda II (n=4) and unknown (n=19). Lineage 3 (Delhi/CAS) was the second most common lineage comprising 44 (14.5%) isolates. Interestingly, six isolates (2%) were belonged to Lineage 7, unique to Ethiopia. Lineage 1 (East-African Indian) and Lineage 2 (Beijing) were represented by 3 and 1 isolates respectively. *M. bovis* was identified in only two (0.7%) TBLN cases. The cluster rate was highest for Ethiopia_3 isolates showing clonal similarity with isolates from North Ethiopia. Lineage 3 was significantly associated with rifampicin resistance.

Conclusions: In TBLN in Southwest Ethiopia, the recently described Ethiopia specific Lineage 4 families were predominant, followed by Lineage 3 and Lineage 4 - Haarlem. The contribution of *M. bovis* in TBLN infection is minimal.

Keywords: Lineage, *Mycobacterium tuberculosis, Mycobacterium bovis,* tuberculous lymphadenitis, Ethiopia

8.2. Introduction

Tuberculosis (TB) is caused by a group of bacteria called *Mycobacterium tuberculosis* complex (MTBc) [1], which comprises closely related species responsible for strictly human and zoonotic TB [2]. The majority of TB in humans is caused by *M. tuberculosis* with a small proportion of TB disease caused by *M. bovis* and *M. africanum* [3]. The MTBc comprises seven human-adapted phylogenetic lineages (Lineage 1 to Lineage 7), with various geographical coverage and frequency, and comprising multiple sub-lineages [4]. Ethiopia is among the 30 high-burden countries for TB, TB/HIV and multidrug-resistant TB (MDR-TB) in the world, with an estimated incidence rate of 192 per 100,000 populations in 2015 [5]. In Ethiopia, extrapulmonary tuberculosis (EPTB) accounts for more than 32% of all forms of TB [6]. Tuberculous lymphadenitis (TBLN) is the most common form of EPTB and accounts for 80% of all EPTB cases [7, 8].

Whether or not the high rate of TBLN in Ethiopia is linked to a specific MTBc lineage has not been systematically investigated. Various findings support the idea of a longstanding host-pathogen co-evolution [9, 10, 11] and found a correlation between human genetic polymorphisms and specific *M. tuberculosis* lineages [12, 13]. Association between MTBc lineages and EPTB have been reported in some studies [12, 14, 15], such as an association between Lineage 3 and EPTB [16]. On the other hand, Coscolla and Gagneux reviewed nearly one hundred published reports and failed to find a clear association between MTBc lineages and TB disease presentation [17].

Several methods for genotyping of MTBc have been developed over the past years [29]. Spoligotyping is the widely used PCR-based reverse-hybridization blotting technique. Spoligotyping is based on polymorphisms in the direct repeat (DR) locus, which consists of 36-bp DR copies interposed by non-repetitive spacer sequence. It is a fast, robust and cost effective genotyping technique, but with limite discrimantory power to distinguish individual strains. In the past few years, a new genotyping technique based on PCR amplification of mycobacterial interspersed repetitive units of variable- number tandem repeats (MIRU-VNTR) was introduced [39]. This method is much faster than the labour-intensive IS*6110* RFLP and requires less DNA. However, MIRU requires more sophisticated equipment such as a sequencer, and therefore is less accessible to most low or middle income countries. Though

24-loci MIRU-VNTR is the recommended method, 15-loci MIRU in combination with spoligotyping can be sufficient for genotyping of MTBc isolates [39].

In Southwest Ethiopia, there is limited information on the genetic diversity of circulating MTBc strains. Recently, a new phylogenetic lineage called Lineage 7 was reported by researchers in Woldiya, Northern Ethiopia [18, 19]. Studies also described new Ethiopian specific families within Lineage 4, namely Ethiopia_2, Ethiopia_3 and Ethiopia_H_{37}Rv-like in multiple sites in Ethiopia [8, 18, 20]. Bovine tuberculosis is an endemic disease in Ethiopia and a recognized problem in cattle with an overall prevalence ranging from 1% to 15% [21, 22, 23, 24] and reaches a high prevalence (up to 50%) in intensive dairy farms [25]. It would seem plausible that zoonotic transmission of *M. bovis* could explain the high prevalence of TBLN. In the present study, we investigated the genetic diversity of MTBc isolates from TBLN patients and the role of newly described Ethiopian specific families and *M. bovis* in this disease presentation.

8.3. Materials and methods

8.3.1. Patients and bacterial isolates

A total of 436 consecutive patients presenting with lymph node swelling from Southwest Ethiopia were tested for TB. These patients were admitted to the Jimma University Specialized Hospital from April 2013 to February 2015. Basic demographic and clinical data were collected using a structured questionnaire. Fine needle aspirate (FNA) was collected from the swollen lymph node of all patients. The first few drops of the aspirates were used for cytomorphological diagnosis and acid fast smear microscopy. The remainder was processed for Xpert MTB/RIF® and culture.

Culture was performed in MGIT960 (Becton Dickinson, USA) and/or on Löwenstein-Jensen (L-J) medium after decontamination by N-acetyl- L-cysteine –NaOH solution at Jimma University-Mycobacteriology Research Center (JU-MRC) as previously described [26]. Primary differentiation of MTBc from non-tuberculous mycobacteria (NTM) was performed by Capilia TB-Neo (TAUNS Laboratories, Japan). Resistance to rifampicin (RIF) was determined by the Xpert MTB/RIF assay (Cepheid, USA) [27]. For 25 patients, Xpert MTB/RIF was not performed due to insufficient sample volume or accidental loss of the sample sediment.

8.3.2. DNA extraction

Each positive liquid culture was subcultured on L-J medium. Two loops of colonies were collected from 4- to 6-weeks-old L-J slants and suspended in 400µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted by heating the isolates at 80°C for 60 min [28] and transported to the ITM, Belgium and Genoscreen, Lille, France for further analysis.

8.3.3. Spoligotyping

Spoligotyping was performed using primers (DRa and DRb) according to the procedure described by Kamerbeek *et al* [29]. The amplified product was hybridized to a set of 43 immobilized oligonucleotides using an in house prepared membrane. Hybridized DNA was detected by the chemiluminescence method (Amersham Biosciences, Little Chalfont, UK) and exposure to an X-ray film (Hyperfilm ECL, AmershamBiosciences). *M. tuberculosis* H_{37}Rv and *M. bovis* BCG were used as positive controls in each run.

8.3.4. MIRU-VNTR typing

A total of 167 isolates were selected for further analysis by 15-loci MIRU-VNTR typing at Genoscreen, Lille, France [30]. These included all isolates belonging to the ill-defined T family (n=116), some of orphans and/or isolates with unusual spoligotype patterns (n=34), 6 isolates with spoligotype patterns of Lineage 7, 1 Beijing strain and 10 rifampicin resistant strains as identified by Xpert MTB/RIF.

8.3.5. qPCR-SNP analysis

Single nucleotide polymorphism (SNP) analysis was carried out at ITM, Belgium, on a selected set of isolates (n=32) representative of the different spoligotypes with their neighboring profile and 6 isolates with spoligotype patterns of Lineage 7. Lineage specific primers and probes were used as previously described [31]. TaqMan real-time PCR was performed according to standard protocols (Applied Biosystems, Carlsbad, USA).

8.3.6. Phylogenetic classification

Spoligotypes in binary format and MIRU-VNTR patterns in 15-digit codes were double entered in an Excel spreadsheet and uploaded on the *MIRU-VNTRplus* data base (www.miru-vntrplus.org). Spoligotypes common to more than one strain were assigned a shared international type number (SIT) according to the updated version of the international spoligotype database SpolDB4 [32]. Spoligotyping patterns and MIRU-VNTR 15-loci profiles

were used to classify the strains into main phylogenetic lineages or families by using the reference strain collection available at *MIRU-VNTRplus* [33]. The strains were classified by the simple match approach that is based on the best match with strains of the reference data base [33, 34] using a cut-off distance of 0.17. In addition, lineage-specific SNP analysis was deployed to assign isolates into major MTBc lineages for those that could not be classified with the above described strategy.

Lineage 7 isolates were defined based on spoligotype patterns, i.e. deletion of spacer 4–24 as previously reported by Firdessa *et al* [19] and high degree of MIRU-VNTR loci similarities with other Lineage 7 strains. Classification of the new Ethiopian specific families were primarily based on characteristic spoligotype patterns: Ethiopia_2 (absence of spacer 13), Ethiopia_3 (absence of spacer 10-19) and Ethiopia_H_{37}Rv-like (absence of spacer 33-36) [8, 18]. In addition, phylogenetic tree based identification was carried out to classify isolates into Ethiopia_2 and Ethiopia_H_{37}Rv-like. A cluster was defined as two or more isolates harboring identical spoligotyping and MIRU-VNTR profiles. The clustering rate was calculated using the following formula: $(n_c - c)/n$, where n_c is the total number of clustered isolates, c is the number of clusters, and n is the total number of isolates.

8.3.7. Ethical considerations

This study was reviewed and approved by Institutional Review Board (IRB) of Jimma University, Ethiopia (Ref. No. RPGC/510/2014) and ITM Institutional Review Board, Antwerp, Belgium (Ref. No. 986/15). For participants less than 18 years of age, assent was obtained, as well as consent from their parent or legal representative.

8.4. Results

Of 436 presumptive TBLN cases who provided FNA samples, 310 were culture positive for MTBc (one isolate per patient). For 305 of these isolates, DNA extraction and spoligotyping were successful. One hundred sixty seven isolates with selected spoligotypes were further analyzed by MIRU-VNTR. One isolate displayed 2 alleles in six independent MIRU-VNTR loci, which suggested a mixed infection and was excluded from further analysis. Another two isolates had no PCR amplicon at one locus, and were treated as missing data at the respective loci in the final analysis. Thus, after excluding 5 isolates with insufficient DNA quality and one isolate with mixed infection, 304 isolates were included in the final analysis (**Figure 1**).

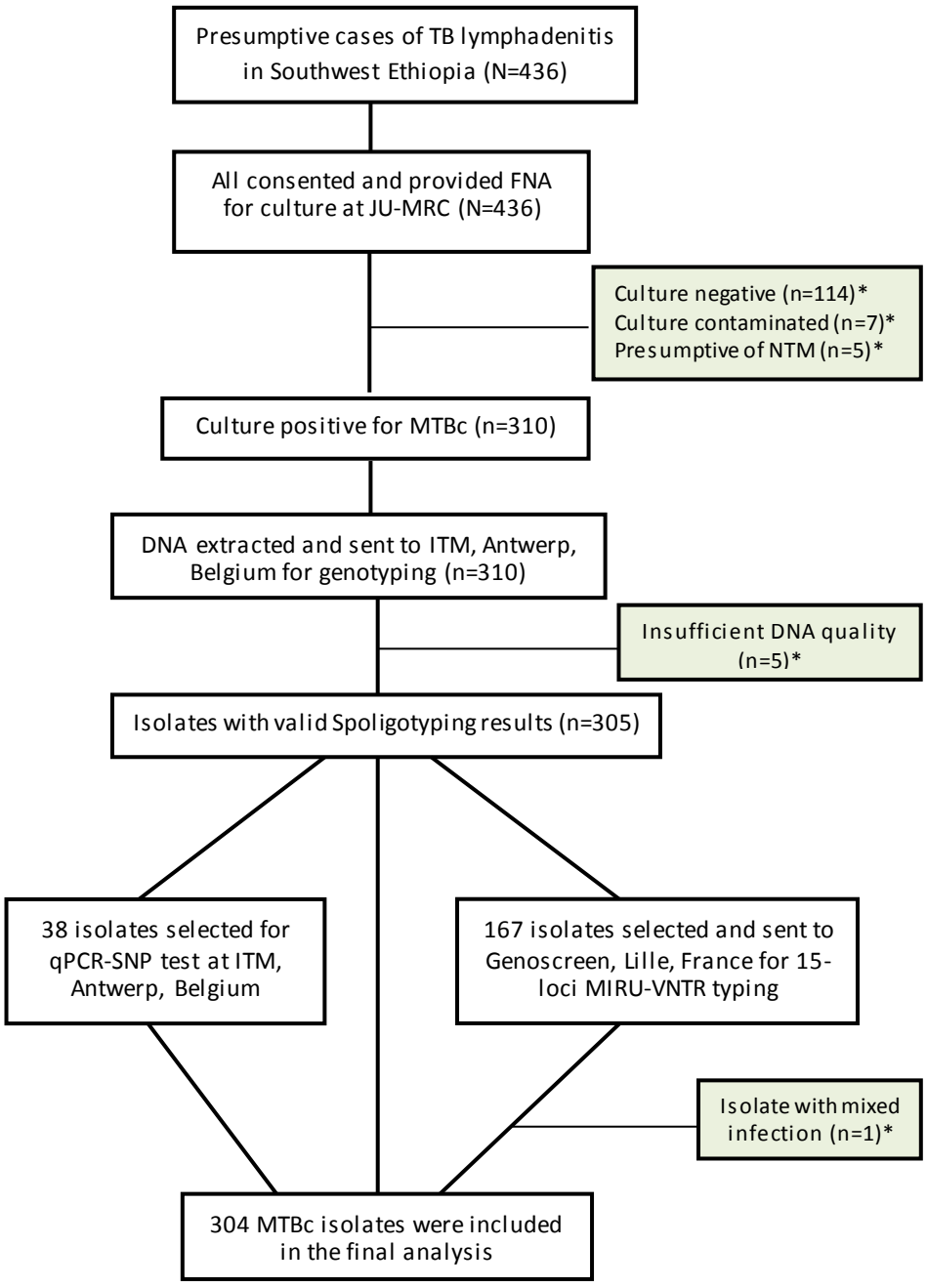


Figure 1: Overall study flow; starting from study participant recruitment, isolation of bacteria and genotyping. FNA: fine needle aspirate, JU-MRC: Jimma University-Mycobacteriology Research Center, MTBc: *Mycobacterium tuberculosis* complex, ITM: Institute of Tropical Medicine, NTM: non-tuberculous mycobacteria, SNP: single nucleotide polymorphism, * excluded from the final analysis

8.4.1. Study participants' characteristics

Of 304 TBLN patients included in the analysis, 53% (161/304) were females (**Table 1**). The patients' mean age was 29 years (±13SD) with a range of 6–78 years. Twenty four (7.9%) TBLN patients were co-infected with HIV. The majority (76.6% (233/304)) of TBLN patients were presented with cervical lymphadenopathy. FNA smear microscopy was positive in only 121 (39.8%) of culture confirmed TB patients. Rifampicin susceptibility data were available for 279 (91.8%) of all strains and revealed 10 (3.6%) RIF-resistant cases.

Table 1: Demographic and clinical characteristics of TBLN patients in Southwest Ethiopia.

Patient characteristics	Total (N=304)	Lineage 1 (n=3)	Lineage 2 (n=1)	Lineage 3 (n=44)	Lineage 4 (n=248)	Lineage 7 (n=6)	*M. bovis* (n=2)
Age							
<15	33(10.9)	1(33.3)	0	5(11.4)	27(10.9)	0	0
15-30	166(54.6)	2(66.7)	0	24(54.5)	134(54.0)	5(83.3)	1(50.0)
31-60	93(30.6)	0	1(100)	12(27.3)	79(31.9)	0	1(50.0)
>60	12(3.9)	0	0	3(6.8)	8(3.2)	1(16.7)	0
Gender							
Male	143(47)	2(66.7)	1(100)	21(47.7)	117(47.2)	2(33.3)	0
Female	161(53)	1(33.3)	0	23(52.3)	131(52.8)	4(66.7)	2(100)
HIV status							
Positive	24(7.9)	1(33.3)	0	4(9.1)	18(7.3)	0	1(50)
Negative	232(76.3)	2(66.7)	1(100)	33(75.0)	191(77.0)	4(66.7)	1(50)
Unknown	48(15.8)	0	0	7(15.9)	39(15.7)	2(33.3)	0
Site of LN							
Cervical	233(76.6)	2(66.7)	1(100)	34(77.3)	189(76.2)	6(100)	1(50)
Axillary	47(15.5)	1(33.3)	0	9(20.5)	36(14.5)	0	1(50)
Inguinal	24(7.9)	0	0	1(2.3)	23(9.3)	0	0
Microscopy							
Positive	121(39.8)	1(33.3)	1(100)	24(54.5)	90(36.3)	3(50)	2(100)
Negative	183(60.2)	2(66.7)	0	20(45.5)	158(63.7)	3(50)	0
RIF status							
Resistant	10(3.3)	0	0	8(18.2)	2(0.8)	0	0
Sensitive	269(88.5)	3(100)	1(100)	33(75.0)	224(90.3)	6(100)	2(100)
Not done	25(8.2)	0	0	3(6.8)	22(8.9)	0	0

Key: Numbers in bracket are reported in %. **Abbreviations**: TBLN= tuberculous lymphadenitis, LN=lymph node, FNA=fine needle aspirate, RIF=rifampicin.

8.4.2. Distribution of different MTBc lineages and families

The population structure showed that 248 (81.6%) isolates belonged to the Euro-American lineage (Lineage 4), with the ill-defined T and Haarlem as the largest families, comprising 116 (38.2%) and 43 (14.1%) isolates respectively. The second most predominant lineage was the Delhi/CAS lineage (Lineage 3) comprising 44 isolates (14.5%). Lineage 1 (EAI), *M. bovis* and Lineage 2 (Beijing) were represented by 3, 2 and 1 isolates respectively. The two *M. bovis* isolates showed a typical bovine spoligotype patterns (SIT 665) lacking spacers 3-5, 9, 16, and 39–43, features that define strains of the African 1 clonal complex of *M. bovis*. Six isolates were identified as Lineage 7 (Ethiopia_1) (**Table 2 and Figure 2**).

Spoligotype analysis revealed 96 different spoligotype patterns: 214 (71.4%) of 304 isolates were assigned to 42 shared types (SITs), whereas 90 (29.6%) isolates exhibited 54 patterns that did not match a SIT in the database (SpolDB4) and were termed orphans (**Table 2**). Among the poorly defined T-isolates, 93% (108/116) of the isolates were classified by MIRU-VNTR*plus* as being part of the newly described Ethiopian specific Lineage 4 families; namely Ethiopia_2 (n=44), Ethiopia_3 (n=34) and Ethiopia_H_{37}Rv-like (n=30). Six of the 'unknown' (U) isolates (SIT910 and SIT1729) in the SpolDB4 were identified as Ethiopia_1 (Lineage 7) (**Table 2**). Nineteen (6.3%) isolates that could not be assigned to previously known or new phylogenetic MTBc families were assigned to the Euro-American lineage after qPCR-SNP analysis. The different lineages/families identified from TBLN patients in Southwest Ethiopia are depicted in **Figure 2.**

Table-2: Spoligotyping patterns, SpolDB4 family and lineages distributions of MTBC isolates (N=304).

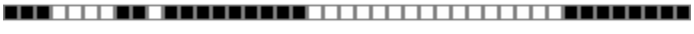
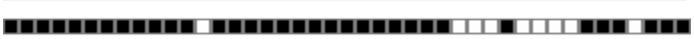
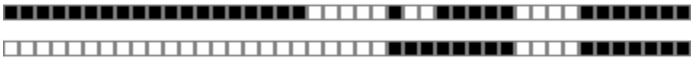
Spoligotype pattern	Major lineage	MTBc family	N
	Bovis	*M. bovis*	2
	2	Beijing	1
	3	Delhi/CAS	2
	3	Delhi/CAS	1
	3	Delhi/CAS	28
	3	Delhi/CAS	3
	3	Delhi/CAS	1
	3	Delhi/CAS	6
	3	Unknown	1
	4	Unknown	5
	4	Haarlem	2
	4	Haarlem	1
	4	Haarlem	1
	4	Haarlem	1
	4	Haarlem	2
	4	Haarlem	1
	4	Haarlem	2
	4	Haarlem	1
	4	Haarlem	14
	4	Haarlem	1
	4	Haarlem	6
	4	Haarlem	1
	4	TUR	1
	4	LAM	1
	4	LAM	1
	4	Ethi_H37Rv-l	23
	4	Ethiopia_2	2
	4	LAM	1
	4	Ugandal	1
	4	Ethiopia_2	1
	4	Ugandal	2
	4	Ugandal	6
	4	Ugandal	2
	4	Ethiopia_2	35
	4	Et_H37Rv-li	5
	4	S	3
	4	Ethiopia_2	1
	4	Ethiopia_3	31
	4	Ethiopia_3	1
	4	Eth_H37Rv-li	2
	4	UgandaII	1
	4	X	2
	4	X	2
	4	X	1
	7	Lineage 7	5
	7	Lineage7	1

Table-2: Spoligotyping patterns, SpolDB4 family and lineages/sublineages (N=304) (Continued…).

Spoligotyping patterns	Major lineage	MTBc family	N
	1	EAI	1
	1	EAI	1
	1	EAI	1
	3	Delhi/CAS	1
	3	Delhi/CAS	1
	3	Delhi/CAS	1
	4	Unknown	1
	4	Haarlem	3
	4	Haarlem	3
	4	UgandaI	2
	4	Unknown	1
	4	Unknown	1
	4	Ethiopia_3	1
	4	Ethiopia_3	1
	4	URAL	1
	4	Haarlem	1
	4	URAL	7
	4	URAL	1
	4	Unknown	1
	4	Unknown	1
	4	Unknown	1
	4	Unknown	2
	4	LAM	2
	4	LAM	2
	4	LAM	2
	4	S	7
	4	S	3
	4	Unknown	1
	4	S	5
	4	S	1
	4	Unknown	1
	4	UgandaI	1
	4	Haarlem	4
	4	Unknown	2
	4	URAL	1
	4	TUR	1
	4	Unknown	1
	4	UgandaII	1
	4	Unknown	1
	4	Unknown	1
	4	UgandaII	1
	4	Unknown	2
	4	Unknown	1
	4	LAM	1
	4	UgandaI	1
	4	TUR	2
	4	URAL	1
	4	LAM	1
	4	LAM	1
	4	URAL	4
	4	Haarlem	1
	4	Haarlem	1
	4	LAM	1

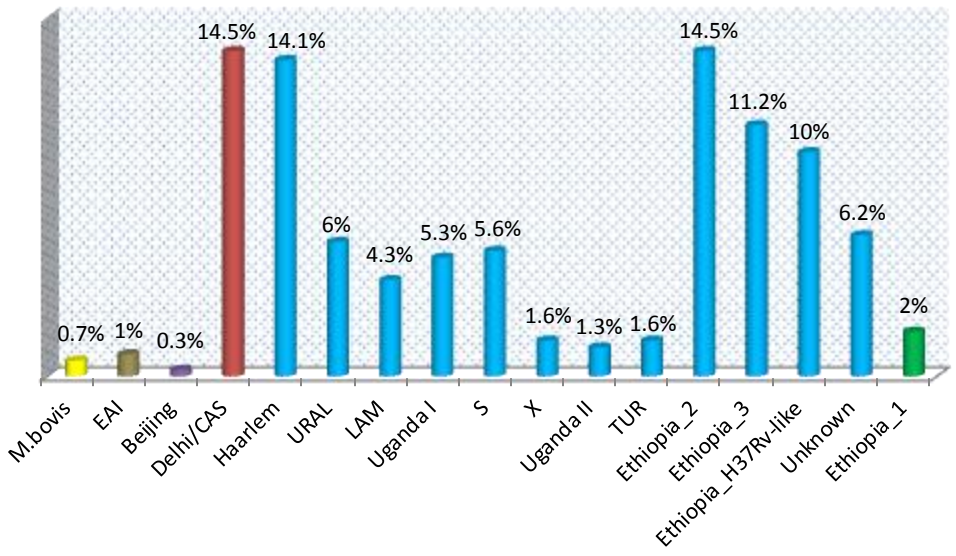


Figure 2: Proportion of MTBc lineages/families identified from TBLN patients in Southwest Ethiopia (N=304) based on spoligotyping, 15-loci MIRU-VNTR and qPCR-SNP analysis. L= lineage, EAI= East-African Indian, LAM= Latin American Mediterranean, CAS=Central Asian, Ethiopia-1= Lineage 7

8.4.3. Cluster analysis

By spoligotyping alone 247 (81.2%) of the 304 isolates were grouped into 49 clusters with each cluster consisting of 2 to 42 isolates. The largest cluster was observed for isolates belonging to the T3 (ST37, Ethiopia_2, n= 42 isolates), followed by strains belonging to T3_ETH (ST149, Ethiopia_3, n=31 isolates), the third largest cluster by strains of the Delhi/CAS lineage (ST25, n = 28 isolates) and the fourth largest cluster by T1 (ST53, Ethiopia_H_{37}Rv-like, n= 25 isolates).

MIRU-VNTR typing of the selected 166 isolates showed highly diverse patterns. One hundred and eight distinct patterns were detected in this collection, including 24 cluster patterns and 84 unique patterns. The highest discrimination was achieved when combining spoligotyping and MIRU-VNTR with the overall clustering rate of 29.5% (**Table 3**). Seventy one of the 166 isolates were grouped into 24 clusters, each cluster comprising 2-17 isolates. MIRU-VNTR analysis was highly discriminatory for the ill-defined T3 families: 42 isolates of T3 (SIT37 and SIT504) were split into 33 different patterns i.e. five clusters comprising 14 isolates with maximum 4 isolates per cluster. Similarly, MIRU-VNTR typing of the 24 T1 (SIT53 and SIT102) isolates showed that 17 isolates produced seven clusters (maximum of 5 isolate per cluster) whereas 7 isolates had unique MIRU-VNTR types. Unlike T3 (Ethiopia_2) isolates, Ethiopia_3

(T3_ETH) isolates were highly clonal and less differentiated by MIRU-VNTR typing. The largest cluster (MLVA MtbC 15-9 type 594-?) containing 17 isolates was formed by Ethiopia_3 (**Figure 3**).

Table 3: Clustering rate analysis by spoligotyping and 15-loci MIRU-VNTR among selected MTBc isolates in Southwest Ethiopia (N=166).

Genotyping methods	No. total of profiles	No. of isolates with unique profiles	No. of clusters	No. of isolates in cluster	Clustering rate (%)
Spoligotyping	43	28	15	138	74.1
MIRU-VNTR	108	84	24	82	34.9
Spoligotyping + MIRU-VNTR	117	95	22	71	29.5

Of ten RIF-resistant isolates that were further typed by MIRU-VNTR, 4 (40%) had unique MIRU-VNTR patterns and the remaining 6 (60%) were clustered into two i.e. one cluster containing 4 isolates (identical spoligotype and MIRU-VNTR patterns) and the other cluster 2 isolates. The majority (80%, 8/10) of RIF-resistant isolates were belonged to the Lineage 3 (Delhi/CAS) (**Figure 4**). Although the numbers are small, the risk of having RIF-resistance was 21.6 fold (95% CI, 4.4–106, *p*-value = 0.001) higher among patients with a Delhi/CAS strains compared to patients with the other. Interestingly, all RIF-resistant isolates harbored mutation at probe E binding site of *rpoB* gene (codon 447-452) as determined by Xpert MTB/RIF test.

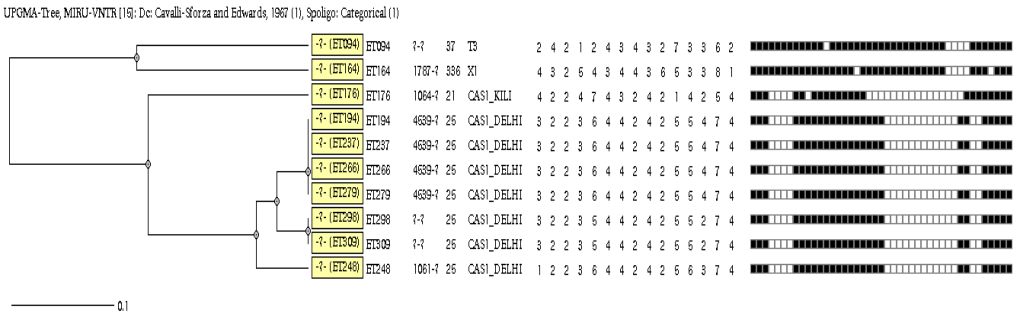
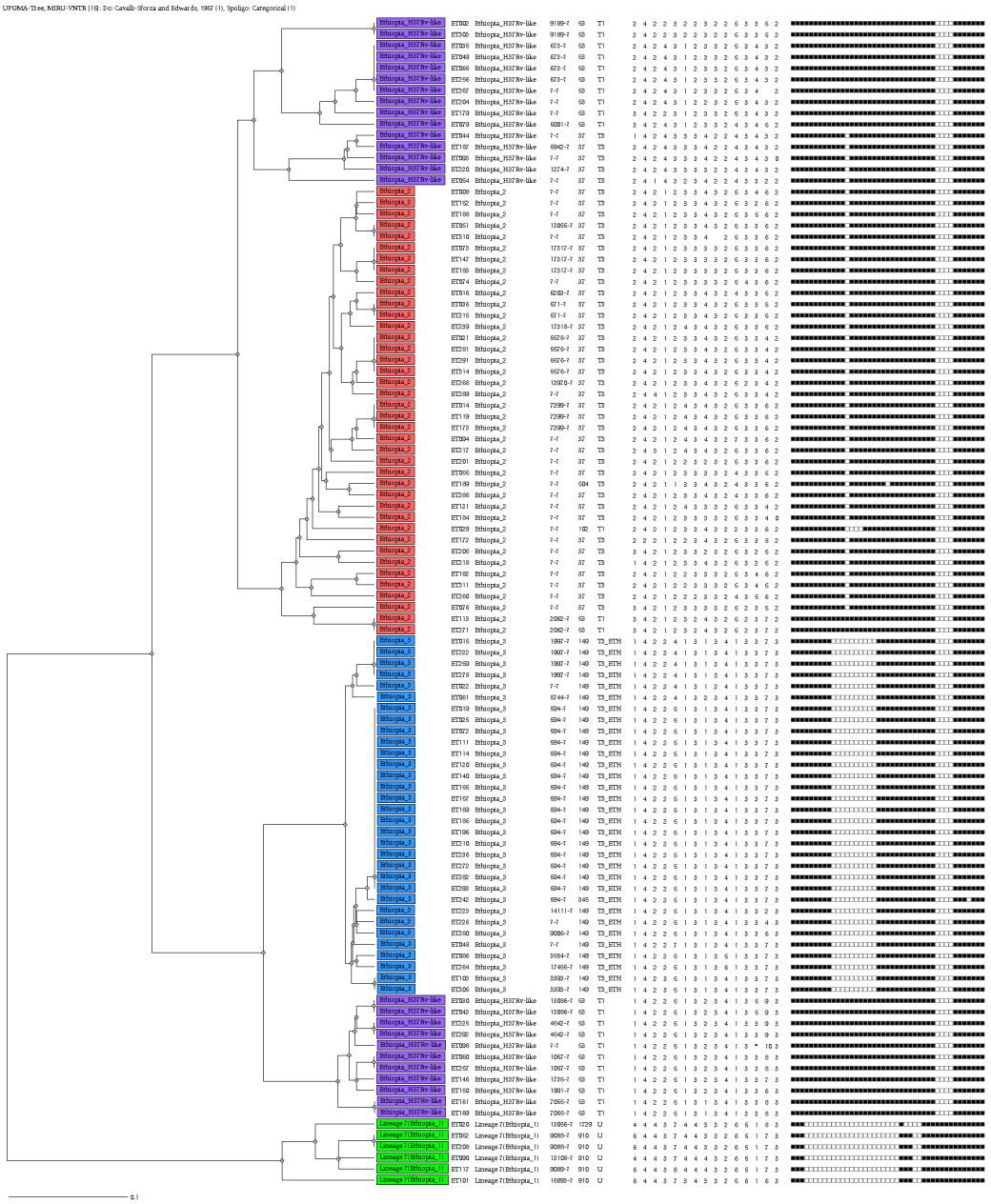


Figure 4: UPGMA dendrogram of rifampicin resistant MTBc isolates from TBLN patients (N=10). The tree was constructed based on spoligotyping and MIRU-VNTR results using MIRU-VNTR*plus* website. Rifampicin-resistance was determined by the Xpert MTB/RIF assay directly on the specimen or decontaminated sediment.



KEY

Ethio_H37Rv-like L4

Ethiopia_2 (L4)

Ethiopia_3 (L4)

Ethiopia_1 (L7)

Figure 3: UPGMA tree of selected Ethiopian specific lineages (n=104) (41 ST37, 31 ST149, 25 ST53, 5 ST910 and 1 each from ST345, ST102, ST504 and ST1729). The tree was constructed based on spoligotyping and MIRU-VNTR results using MIRU-VNTR*plus* website. The color indicates the phylogenetic lineage/family to which Ethiopian specific MTBc lineages/families belong. Ethiopia_3 is homogeneously constituted by T3_ETH genotypes; Ethiopia_2 contains genotypes from T3 and T1; Ethiopia_H$_{37}$Rv-like contains genotypes from T1 and T3; Lineage 7 (Ethiopia_1) contains genotypes from U.

8.5. Discussion

The high rate of TBLN in Southwest Ethiopia is not explained by bovine TB transmission, and the majority is caused by Ethiopian specific families in Lineage 4 of the MTBc. The MTBc lineage distribution from TBLN patients in our study is similar to the distribution of strains documented previously from both pulmonary and TBLN patients in different regions of Ethiopia [7, 19], reflecting the absence of pathogen-specific genetic factors associated with the high rate of TBLN in Ethiopia. Our finding is also in line with the report from Coscolla & Gangeux [17] who found no clear association between genotypic variation and clinical phenotypes. Ethiopian specific families within the Lineage 4 also found to cause TBLN in Northern Ethiopia [8], where Lineage 7 is more common. It is of note that in our dataset some 'typical' spoligotype profiles of Ethiopia_2 and Ethiopia_H_{37}Rv-like were interspersed on the phylogenetic tree when combining spoligotyping & MIRU-VNTR (**Figure 3**). Considering spoligotyping alone, Ethiopia_2 (T3) isolates differed from Ethiopia_H_{37}Rv-like (T1) because the former lacks spacer 13. Isolates of Ethiopia_2 and Ethiopia_H_{37}Rv-like were genetically diverse and some of them were ambiguously classified even after MIRU-VNTR 15-loci analysis.

Lineage 3 (Delhi/CAS) is the other dominant lineage in this study. Previous studies [8, 18, 20] also confirmed the presence of Delhi/CAS across the different regions of Ethiopia, indicating the successful spread of these strains through human movement, either from Central Asia to Ethiopia or the other way around, which would be in agreement with the "out of Africa" theory postulated by Gagneux and colleagues [10]. This hypothesis could be tested by genome sequencing of Asian and Ethiopian Lineage 3 isolates. In contrast to North Ethiopia where Delhi/CAS is the most prevalent lineage [8, 18], a shift towards Ethiopian specific Lineage 4 strains was observed in our study. Interestingly, Lineage 3 (Delhi/CAS) was found to be significantly associated with infection of rifampicin resistant *M. tuberculosis.* As shown in **Figure 4**, six of the eight RIF-resistant -Lineage 3 isolates formed two clusters (the first cluster with 4 isolate and the second with 2 isolates) and shared their RIF-resistance conferring mutations at *rpoB* gene (codon 447-452), thus possibly suggesting recent transmission of RIF-resistant strains.

We identified Lineage 7 in six (2%) TBLN patients in Southwest Ethiopia, similar to the 2% observed among pulmonary TB patients from South and East Ethiopia, but lower than the 8-10% reported among both pulmonary and TBLN patients in the North Eastern highlands of

Ethiopia [8, 18, 19]. Lineage 7 has previously been reported in Ethiopia [19, 35] and among Ethiopian immigrants in Djibouti [36], yet not elsewhere. Like *M. africanum* (lineage 5 and 6) that is localized in West Africa, Lineage 7 is limited to the Horn of Africa. The restricted geographic distribution suggests Lineage 7 strains are not as successful as modern lineages, requiring particular conditions that limit their spread. Possible explanations for this geographical restriction could be that Lineage 7 has a lower rate of progression to disease relative to other lineages [37], with subsequent out competition by other MTBc lineages, or Lineage 7 strains have a host preference for ethnically Ethiopian people. In our study, we could not obtain detailed information on the ethnic background of the participants. Phylogenetic tree analysis clearly showed that Lineage 7 strains were located far from the recently described Ethiopian clades and is of considerable evolutionary interest because it represents a phylogenetic branch intermediate between the ancient and modern lineages of *M. tuberculosis* (**Figure 3**). However, further investigation of Lineage 7 is warranted to explain the virulence factors, pathogenesis and clinical presentations of TB disease.

A report by Kidane *et al.* hypothesis that the exceptionally high incidence of TBLN in Ethiopia could be due to zoonotic transmission of *M. bovis* from cattle [38]. In our study, *M. bovis* was identified only in two (0.7%) TBLN patients, similar to findings of another recent study in Ethiopia where 0.4% *M. bovis* was reported among pulmonary TB patients and none among TBLN patients [19]. Our data indicate that the overall contribution of *M. bovis* to human TBLN is minor in Southwest Ethiopia. In general, declining rates of *M. bovis* isolation from human TB patients have been reported in Ethiopia, despite continued consumption of unpasteurized milk and milk products, which is thought to have a high risk for human infection with *M. bovis* in a setting with a high prevalence of bovine tuberculosis.

The overall clustering rate in this study was found to be 29.5%. This is in agreement with previous reports from Ethiopia in TBLN [8] and pulmonary TB patients [20] that showed clustering rates of 35% and 31.2% respectively. The cluster rate in our study was significantly lower than the rate reported in Northwestern Ethiopia from pulmonary TB patients (45%) [18], which could be due to the fact that TBLN patients are thought to be non-infectious, although the selection of isolates for MIRU-VNTR likely introduced bias in the clustering estimate. However, even in TBLN patients, the clustering remains very high for Ethiopia_3 isolates which is in agreement with another study in Northern Ethiopia [8]. Seventeen of our Ethiopia_3

isolates were clustered (identical spoligotype and 15-loci MIRU-VNTR profiles) with ten Ethiopia_3 isolates from Northern Ethiopia [8]. This indicates that Ethiopia_3 strains remain the predominant source of the most recent infection for TBLN cases, though information about possible epidemiological links between patients with clustered isolates was not available.

This study has some major limitations. First, MIRU-VNTR analysis was restricted to a selected set of MTBc isolates and 15-loci due to financial constraints, limiting the discriminatory power. Secondly, our study only included clinical cases visiting Jimma University Specialized Hospital with potential bias towards the overall TB population of this region, and epidemiological investigations of contacts, particularly among patients with clustered isolates, could not be performed. Nonetheless, our study provides valuable information on the etiology of TBLN in Southwest Ethiopia.

8.6. Conclusions

This study revealed a high diversity of circulating MTBc lineages responsible for TBLN in Southwest Ethiopia. The Ethiopian sub-lineages are the most dominant in TBLN patients followed by Delhi/CAS and Haarlem, yet disease presentation does not seem to be linked to the lineage type. We reported the first presence of Lineage 7 among TBLN patients in Southwest Ethiopia. Zoonotic transmission of *M. bovis* infection has been excluded as a major factor in TBLN. A more detailed genetic analysis by whole genome sequencing is warranted to clearly define the genetic variants of the poorly defined locally contained Ethiopian families.

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Chapter nine: General discussion

9.1. TB diagnostic challenges

The main obstacle for TB control is the lack of adequate tools for TB diagnosis. Failure to control the spread of TB is largely due to our inability to detect and treat all infectious cases in a timely manner, causing continued transmission within communities. In many regions of the world, TB case detection rates remain at unacceptable levels. In the WHO Africa Region, about 40% of active TB cases remain undetected and continue to transmit *M. tuberculosis* [1]. Furthermore, less than 50% of the estimated 500 000 new MDR-TB patients each year are detected, most of them following prolonged diagnostic delay [2]. Failure to detect drug resistance results in inappropriate treatment and premature death of the individual patient, but it also facilitates amplification of resistance and ongoing transmission within the community [3].

The most commonly used diagnostic tests for TB are laboratory based, and multiple investigations may be necessary over a period of weeks or months before a diagnosis is made [4]. In well-developed countries, TB diagnosis by smear microscopy is usually confirmed by culture, followed by the identification of the MTBc strain and DST. Sensitivity is also enhanced by using advanced imaging techniques, rapid NAATs, induced sputum, or invasive techniques such as bronchoscopy with lavage and tissue biopsies [5]. However, many of the patients in resource poor regions of the world have no access to these technologies.

In resource-limited settings, TB control relies on passive case finding among individuals self-presenting to health care facilities, followed by either diagnosis based on clinical symptoms or laboratory diagnosis using sputum smear microscopy. The limitations of the existing TB diagnostics contribute to diagnostic delays with serious consequences for public health efforts to control the epidemic [4, 6]. The vast majority of TB suspects in endemic countries present to peripheral healthcare facilities that may have no electricity and limited or no laboratory facilities. Childhood TB, sputum smear–negative pulmonary TB, EPTB and drug-resistant TB remain the greatest diagnostic challenges [6]. High-priority must be given for determining the feasibility of using accurate, rapid, low-cost, easy to interpret diagnostic methods that require minimum infrastructures.

Over the past decade, several new tests have become available for detecting active TB disease and identifying drug-resistant TB [7, 8] and WHO has endorsed many of these technologies. The majority of these have been molecular- or culture-based technologies that require considerable laboratory infrastructure. Some of the technologies are not affordable or can only function under referral laboratory conditions, and are unlikely to reach the mass of undiagnosed TB cases in the high-burden countries. Although new diagnostic methods are being developed, application of new technology in developing countries has been slow.

9.2. Xpert MTB/RIF

The most significant advance toward a POC test for TB has come in the field of NAATs with the development of the Xpert MTB/RIF assay [9, 10]. It is a self-contained, fully integrated, automated platform that can be used with minimal technical skills and no advanced biosafety equipment is needed. These characteristics of Xpert MTB/RIF make it suitable for lower biosafety level laboratories and even for use in near POC settings. Since the WHO's endorsement in 2010 [11], there has been a surge of donor funding and an establishment of a concessionary pricing scheme for low and middle income countries (LMICs) to facilitate the global roll-out of Xpert, particularly in high TB-burden countries [12, 13]. In line with the WHO recommendations, the Ethiopian Federal Ministry of Health, in 2014, has recommended starting roll-out of the Xpert MTB/RIF in selected settings of Ethiopia to improve TB diagnosis and detection of drug resistance. Despite these efforts, there still are considerable gaps in Xpert MTB/RIF diagnostic coverage in Ethiopia, and routine diagnostics continue to rely on smear microscopy and clinical diagnosis.

Although Xpert MTB/RIF had been approved by WHO at the time of this study, there are several reasons why Xpert MTB/RIF needed to be further evaluated: 1) there were very little data on how the test performs in Ethiopia, where about one third of TB cases are smear-negative; 2) Although Xpert MTB/RIF sensitivity was reported to be modest (55-86%) in smear-negative sputum specimen, no data existed on the effect of treating sputum with bleach followed by simple centrifugation on the sensitivity of Xpert MTB/RIF when the test is placed in peripheral health centers with limited facilities; 3) Xpert MTB/RIF has been initially endorsed and optimized for sputum specimens and there was dearth of published data on the performance of Xpert MTB/RIF for the diagnosis of EPTB; 4) In Ethiopia, a country that has an exceptional high rate of EPTB (>35% of TB cases) it seemed not cost effective to test every

presumed EPTB case by Xpert MTB/RIF; 5) Most previous studies assessed Xpert MTB/RIF performance using culture alone as a reference standard which is known to be suboptimal for EPTB, only few compared Xpert MTB/RIF with composite reference standard including microbiological and clinical data.

9.3. Alternative approaches for TB diagnosis

Evaluation of alternative TB diagnostic strategies is a global priority. Specimen preparation is an integral part of the diagnostic process. One alternative approach is improving the specimen collection and processing to increase the sensitivity of the existing methods by simple and efficient ways affordable in resource poor settings. Pre-treatment of paucibacillary clinical samples (smear-negative and extra-pulmonary samples) with either bleach or NALC-NaOH followed by centrifugation could improve the performance of the conventional (smear microscopy) and new methods (such as Xpert MTB/RIF). Another approach has been to improve the technologies we already have, making them easier to use. For pulmonary TB, WHO has recommended that conventional fluorescence microscopy be replaced by LED microscopy in all settings where fluorescent microscopy is used and that LED microscopy be phased in as an alternative for conventional ZN microscopy in both high- and low volume laboratories [14, 15]. Following the WHO recommendation, the Ethiopian Federal Ministry of Health has started rolling out LED- microscopy in TB laboratories throughout Ethiopia. However, little was known about the role of LED-microscopy for the routine diagnosis of TBLN in Ethiopia.

9.4. TB diagnostic evaluation

A major challenge in TB control is the lack of an accurate, cost-effective, widely available and POC test. The development and evaluation of such test is on top of the research priority list. Diagnostic research is an important area of study that requires a highly multidisciplinary approach to evaluate a test's accuracy, applicability and feasibility, and clinical and economic impact. The current theoretical concept for development and endorsement of TB diagnostics includes smaller clinical evaluation trials preceding larger demonstration studies, which are conducted at multiple sites to assess performance characteristics and early patient-important outcomes. Evidence from these studies is being analyzed and a group of experts gives recommendations to the WHO's Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) after a new diagnostic tool has been thoroughly assessed through systematic

reviews and meta-analysis based on GRADE (Grading of Recommendations Assessment, Development and Evaluation) standards [16, 17]. Evidenced-based recommendations are eventually proposed to support scale-up at country.

More recently, a two-step process has been proposed in order to make endorsement and scale-up of diagnostic tools. The first step starts with a technical recommendation, based on accuracy data and some cost and feasibility data, while the second is to make wider programmatic recommendations focused on patient-important outcomes, cost-effectiveness and operational issues in routine use [18]. This knowledge will be vital in providing directions for policy decision and guideline development.

In this thesis, we discuss three important areas in which progress is sought. The first is to improve the diagnosis of smear-negative PTB by optimizing the use of Xpert MTB/RIF: we evaluated the added value of testing multiple sputum samples and the effect of bleach pre-treatment followed by centrifugation on the sensitivity of Xpert MTB/RIF. The second is to identify a rapid, accurate, affordable and simple to use near-POC test for diagnosing EPTB (particularly TB lymphadenitis which accounts for 80% EPTB) in Ethiopia. The third is to investigate whether the exceptionally high rate of TB lymphadenitis in Ethiopia is linked to a specific genotype of MTBc strains.

9.5. Diagnosis of smear-negative PTB

In resource-constrained settings where sputum culture and NAATs are not routinely available, diagnosis of PTB is based on physical examination, clinical signs, sputum smear microscopy and chest X-ray radiography. In chapter three (**Paper I**) of this thesis, we described the diagnostic performance of Xpert MTB/RIF for the diagnosis of smear-negative PTB; one of the first studies in Ethiopia. This study was not limited to evaluating the diagnostic performance, but also assessed the incremental yield of testing more than one sputum specimen and the effect of a simple sample preparation procedure using bleach followed by centrifugation on the sensitivity of Xpert MTB/RIF in high TB burden settings.

Our study suggests that Xpert MTB/RIF out performs smear microscopy and significantly increased the detection rate of smear-negative PTB cases. The incremental yield of performing the second Xpert MTB/RIF test per patient was insignificant. Hence, in a high TB burden country like Ethiopia, repeating the Xpert MTB/RIF test would significantly increase the

laboratory workload and expenses with little or no added diagnostic value. This is in contrast to the overall yield of smear microscopy which was reported to be superior with multiple sputum specimens than with a single specimen [19]. **However, in our study, bleach pre-treatment of smear-negative, culture-positive sputum followed by centrifugation significantly increased the sensitivity of Xpert MTB/RIF, though its usefulness in detecting culture-negative patients need further study.**

Compared to culture, Xpert MTB/RIF provided rapid results with modest sensitivity (63.2%) and excellent specificity (99%) in AFB-negative sputum specimen. This gives the clinician sufficient confidence to initiate anti-TB treatment when the Xpert MTB/RIF result is positive, although a negative Xpert MTB/RIF result does not exclude a diagnosis of smear-negative PTB. Patients with a strong clinical possibility of PTB despite a negative Xpert MTB/RIF result should be started on anti-TB treatment. Hence, it is important to ensure that clinicians are aware of the Xpert MTB/RIF limitations during its implementation and routine clinical practice.

In routine clinical practice, Xpert MTB/RIF test has distinct advantages. Patients can potentially be diagnosed with TB at their first visit, providing same-day diagnosis which could potentially limit loss to follow up during diagnostic evaluation of smear-negative PTB patients, which would possibly shorten the time to treatment and reduce disease transmission. The other advantage is that Xpert MTB/RIF can be performed by a technician with minimal training at the same level of biosafety as microscopy. Moreover, the ability of Xpert MTB/RIF to identify rifampicin resistance is a further asset of the device. However, larger scale implementation and cost-effectiveness studies should be carried out in Ethiopia, to identify the true benefits over conventional methods in the time to treatment initiation and subsequent benefits in clinical outcomes.

9.6. Diagnosis of tuberculous lymphadenitis (TBLN)

TBLN is the most common form of EPTB in Ethiopia, accounting for approximately 80% of EPTB cases. TBLN usually presents as a slowly progressive, painless swelling of lymph nodes and the majority of cases have no active lung involvement [20, 21]. The diagnosis of TBLN is particularly challenging in low-income countries, where facilities for mycobacterial culture are rare and difficult to access. The differential diagnosis is wide and includes infections, neoplasms, non-

specific reactive lymph node hyperplasia, sarcoidosis and connective tissue diseases. In Ethiopia, the diagnosis of TBLN is routinely made by FNA cytology and AFB smear microscopy.

FNA cytology has emerged as a first-line diagnostic technique, and is sensitive, less invasive, and more practical than biopsy, especially in resource-limited settings. However, FNA cytology has limited specificity because of the presence of some cytologic components in lesions other than those associated with TB [22]. Conventional AFB stain result has excellent specificity for TBLN, though the sensitivity is quite low, ranging from 20-43%, due to the paucibacillary nature of FNA samples [23, 24]. Thus, in three of our studies (paper II, paper III and paper IV) we evaluated the potential utility of conventional smear-microscopy, LED-fluorescence microscopy and Xpert MTB/RIF for diagnosing TBLN in Ethiopia.

Chapter four (**Paper II**) of this thesis describes the added value of pretreating and concentrating FNA specimen on the sensitivity of smear-microscopy for diagnosing TBLN. A wide variety of specimen processing protocols has been described for TB diagnosis. N-acetyl-L-cysteine (NALC) is most commonly used for liquefaction, with sodium hydroxide (NaOH) for decontamination [25]. In our study, all FNA specimens were treated by NALC-NaOH followed by centrifugation. The concentrated sediment was used for culture and AFB smear microscopy. **This study has documented improved sensitivity of conventional AFB smear microscopy for the diagnosis of TBLN using concentrated FNA sample.** Though most previous studies showed controversial findings on the incremental yield of concentration methods, we clearly demonstrated that the sensitivity of ZN smear microscopy was significantly increased after concentration in our setting. The sensitivity of ZN was 34.6% on direct smear and it increased to 66% on concentrated smear. The increased sensitivity by the concentration method could be due to NALC processing and centrifugation. This has been reported to facilitate identification of bacilli by providing a clearer microscopy field through digestion of debris and concentrating bacilli through subsequent centrifugation [26]. We also reported higher grade of AFB positivity by the concentration method compared to direct method, making the bacilli easily visible within a shorter screening time.

Although the sensitivity of AFB smear microscopy significantly improved after concentration compared to direct microscopy, it still remains suboptimal. Thus AFB smear microscopy - even after concentration of FNA samples - cannot be a stand-alone diagnostic tool for TBLN. In areas

where culture facilities and rapid molecular tools are not available, cytological diagnosis can be used in conjunction with the concentration method for better management of TBLN patients. Moreover, specimen processing procedures involving centrifugation requires a refrigerated centrifuge with sealed safety cups and aerosol tight rotors and a biological safety cabinet (BSC). The cost, installation and maintenance of these instruments may be challenging for laboratories in resource-poor settings. Further studies are warranted to evaluate biosafety, training, turnaround times, and financial issues related to the implementation of a concentration step in AFB smear microscopy in peripheral laboratories.

Chapter five (**Paper III**) of this thesis describes the diagnostic accuracy of fluorescence microscopy using light-emitting diodes (LED) on routinely collected FNA specimens from TBLN presumptive cases. Despite WHO recommendation on the use of LED-microscopy, it has been used infrequently in clinical practice for routine diagnosis of TB in Ethiopia. Moreover, the clinical utility of LED- microscopy for the diagnosis of TBLN is not well investigated. In paper III, smear was made from unprocessed sample and examined by LED-microscopy. **We found that LED- microscopy has higher sensitivity and similar specificity compared to ZN microscopy.** LED-microscopy identified 20% of culture confirmed TBLN cases missed by conventional ZN microscopy. LED-microscopy will most benefit high-workload clinics in high-burden TB countries like Ethiopia by reducing the time required to screen slides. However, upon implementation of LED-microscopy, its impact on trends in case detection, patients' outcomes and performance in routine use should be assessed. Moreover, a country that implemented LED microscopy should adopt WHO-endorsed internal and external quality assurance programs into their quality assurance program.

There was evidence that sample pretreatment with subsequent concentration enhances the sensitivity of Xpert MTB/RIF for the detection of TB in clinical specimens [27] although optimized sample processing protocols are not available. In chapter six (**Paper IV**) of this thesis we evaluated the performance of Xpert MTB/RIF for the diagnosis of TBLN on concentrated FNA sample in Southwest Ethiopia. A total of 143 FNA samples were processed by NALC-NaOH method and centrifuged. The concentrated pellets were used for culture (L-J) and Xpert MTB/RIF. The diagnostic accuracy of Xpert MTB/RIF was calculated against a composite bacteriological methods (culture and/or smear microscopy).

The findings of paper IV indicate excellent performance of Xpert MTB/RIF for the diagnosis of TBLN on concentrated FNA sample (sensitivity 88% and specificity 91%). Besides improved sensitivity, the Xpert was able to identify patients with TBLN due to rifampicin-resistant TB. In contrast to conventional tools, Xpert MTB/RIF can differentiate MTBc from NTM. Our data clearly indicate that Xpert MTB/RIF has superior performance compared to FNA cytology, direct and concentrated ZN microscopy and LED-microscopy, and significantly increased the relative proportion of diagnosed TBLN cases, allowing rapid initiation of anti-TB treatment.

In addition, Xpert MTB/RIF is an easy, rapid and suitable method to be used in TB endemic settings and may be the optimal first-line test for the diagnosis of TBLN and merits consideration for implementation in routine clinical practice. Despite the fact that Xpert MTB/RIF performed better on processed FNA specimens (paper IV), this procedure requires a refrigerated centrifuge and BSC. Adding in such processing steps would restrict the use of Xpert MTB/RIF to secondary and tertiary care settings, jeopardizing its implementation at peripheral healthcare settings. Remarkably, in our paper V (unpublished data) presented in chapter seven, we also reported excellent sensitivity (90%) of Xpert MTB/RIF on unprocessed FNA specimen.

Although Xpert MTB/RIF certainly represents an important advance in TBLN diagnosis, some logistical challenges may prevent it from being the ultimate POC test. Among such challenges are the need of stable electric power, the cartridge supply, storage conditions and the cost. Even though the cost of the cartridge was subsidized by an agreement involving FIND and the manufacturer, cost can still be a problem for the widespread scale-up of the assay. There is still a need for technologies operating with little or no laboratory infrastructure and potentially representing an important step towards the creation of a real POC diagnostic test for TB, including TBLN.

In four of our studies (paper I, II, II and IV), culture alone was used as a reference standard. However culture may miss cases of smear-negative or extrapulmonary TB due to the paucibacillary nature of the disease. Moreover, studies demonstrated the higher complexity of mycobacterial populations in sputum, distinguishable by specific growth requirements, and collectively designated as differentially culturable tubercule bacteria (DCTB) [37]. More recently Rosser and colleagues demonstrated culture-supernatant dependent DCTB in EPTB

cases, questioning the use of culture alone as arference standard in extrapulmonary specimens [38]. Thus, culture alone may not perfectly correspond to true target disease status, and estimates of the accuracy of the test under study (index test), such as sensitivity, specificity, and predictive values can be biased. One method to reduce this bias is to use a composite reference standard.

9.7. Xpert MTB/RIF for diagnosing different forms of EPTB

EPTB is a complex and multifaceted disease which constitutes a significant proportion of the global TB burden. Lack of simple and accurate POC test remains one of the major gaps in EPTB care and control. A prompt identification and treatment of cases is required to reduce morbidity, mortality and disability. A high index of clinical suspicion is required for laboratory evaluation as the disease presents in various ways. Because of the involvement of obscure inaccessible sites, invasive procedures may have to be employed to obtain adequate sample amounts or volumes of body fluids/tissue for analysis. The paucibacillary nature of disease and non-uniform distribution of microorganisms may lead to false negative results for most of the laboratory procedures [28].

Excision/aspiration biopsy of tissue with routine histological/cytological analysis showing granulomatous inflammation plays a central role in the diagnosis with its limitation of wide differential diagnoses. Culture has always been considered the 'gold standard' for EPTB diagnosis. However, culture - even using liquid medium - is not the ideal test for EPTB. To better assess diagnostic tests, a composite reference standard is increasingly used which integrates microbiology, clinical findings, other test results and follow-up evaluations to optimize the definition of TB cases. The combined reference standard finds its highest added value over microbiology only in particular 'difficult' forms of TB like smear-negative TB and EPTB [28, 29]. A major challenge of the composite reference standard is the lack of criteria for standardization: different combinations of clinical findings, X-ray, histology/cytology, microbiology and response to treatment are used in different studies with non-uniform case definition among studies [30].

Xpert MTB/RIF has been developed and widely implemented in low-income countries for the diagnosis of patients at risk of MDR-TB and HIV associated TB [11]. More data is required to elucidate the performance of Xpert MTB/RIF in non-respiratory samples within subgroups at

high risk of EPTB. Effort should also be undertaken to optimize the reference standard for evaluation of Xpert MTB/RIF and other new diagnostics in EPTB. In chapter seven (**Paper V**) of this thesis, we evaluated the diagnostic accuracy of Xpert MTB/RIF for the diagnosis of patients suspected of having EPTB by using different types of specimen. In this study, Xpert MTB/RIF results were compared to a CRS which is composed of liquid culture (MGIT 960), microscopy, cytology and radiological findings and treatment response.

The findings of paper V show that Xpert MTB/RIF can be applied to extra-pulmonary specimens with high specificity (pooled specificity of 98%), highlighting its utility as a rule-in test for EPTB diagnosis. This gives the clinician sufficient confidence to initiate TB treatment when Xpert MTB/RIF test is positive. **The Xpert MTB/RIF sensitivity was extremely heterogeneous, (30% in fluids and 90% in lymph nodes specimens) and thus maximum benefit can be obtained by targeting specimens that give better yield.** Thus**,** Xpert MTB/RIF may be used as initial diagnostic test for testing lymph node specimens from patients suspected of having TBLN. While Xpert MTB/RIF has modest sensitivity in CSF, it could significantly improve the diagnosis of TB meningitis in places where culture or more sensitive tests are not available. One of the key advantages of Xpert MTB/RIF is that it is a rapid diagnostic test, potentially providing a result in two hours. This is a particularly important feature of the test in life-threatening forms of EPTB, such as TB meningitis.

Xpert MTB/RIF has low sensitivity in fluids (30%-32%) and was negative in a significant number of patients with probable pleural or peritoneal TB cases, indicating that Xpert MTB/RIF cannot be used reliably to rule out TB in fluids (such as pleural and peritoneal). In such cases, clinical diagnosis remains an essential part of the diagnostic pathway until better and more sensitive tools are available. Centrifugation of paucibacillary fluids (such as pleural) at higher speed (3500g) is supposed to increase the sensitivity of Xpert MTB/RIF, although not attempted in our studies. Studies show that Xpert MTB/RIF greatly accelerated the time to diagnosis, with a median time of 0 days compared with 1 day for smear microscopy, 16 days with liquid culture, and 20 days with solid culture [31]. Since most EPTB cases are smear-negative, a positive culture is the only confirmatory test, and this can mean a delay of 2 -3 weeks before correct treatment can be initiated. On the other hand, patients suspected to have some forms EPTB may sometimes be hospitalized in isolation rooms until cultures come back negative. This

possesses unnecessary hospitalization, prolongs wait times, and causes considerable medical, social and financial burden on hospitals and to the health system.

Table 1: Summary methods evaluated in this thesis and their clinical utility

Diagnostic method	Clinical utility of tests
Direct microscopy (sensitivity =25-32%, specificity = 94%-100%)	Simple, inexpensive, rapid and specific. Low sensitivity, especially for extrapulmonary TB
Cytology (sensitivity = 80-88%, specificity = 50% -58%)	Sensitive, less invasive, and more practical than biopsy. However, cytology has limited specificity and requires highly trained pathologist which is the case in Ethiopia who are not available at relatively lower level health facilities.
Xpert MTB/RIF for smear-negative PTB Unprocessed (sensitivity= 63%) Bleach processed (sensitivity = 73.8%)	Xpert MTB/RIF on bleach processed or unprocessed sputum specimen can improve the diagnosis of smear-negative TB but patients with a strong clinical possibility of PTB despite a negative Xpert MTB/RIF result should be started on anti-TB treatment. Hence, it is important to ensure that clinicians are aware of the Xpert MTB/RIF limitations during its implementation and routine clinical practice.
Smear microscopy (ZN) (processed specimen) for TB lymphadenitis (sensitivity = 61%, specificity= 91%)	Concentrating lymph node specimen significantly improved the sensitivity of microscopy. However, specimen processing procedures involving centrifugation requires a refrigerated centrifuge and aerosol tight rotors and a biological safety cabinet, which questioned the implementation of this method in resource poor settings.
LED-Fluorescent microscopy (unprocessed specimen) for TB lymphadenitis (sensitivity = 46%, specificity= 90%)	More sensitive and requires shorter screening time compared to conventional bright filed microscopy but suboptimal sensitivity. In setting where there is no Xpert MTB/RIF, LED-microscopy can be supplemented with cytology for better management of patients suspected of having TB lymphadenitis.
Xpert MTB/RIF (processed specimen) for TB lymphadenitis (sensitivity = 88%, specificity= 91%)	Xpert MTB/RIF has superior performance compared to cytology, direct and concentrated ZN microscopy and LED-microscopy, and significantly increased the relative proportion of diagnosed TB lymphadenitis cases, allowing rapid initiation of anti-TB treatment. However, the specimen processing step we used in this study would restrict the use of Xpert MTB/RIF to secondary and tertiary care settings, jeopardizing its implementation at peripheral settings.
Xpert MTB/RIF (unprocessed specimen) for different types of EPTB compared to CRS; Sensitivity for lymph nodes specimen=90%, Sensitivity for CSF= 53% Sensitivity for pleural = 30% Sensitivity for peritoneal = 32%	Xpert MTB/RIF may be used as initial diagnostic test for testing lymph node specimens from patients suspected of having TBLN. While Xpert MTB/RIF has modest sensitivity in CSF, it could significantly improve the diagnosis of TB meningitis as it provides rapid results. A negative Xpert MTB/RIF on fluid specimens such as pleural and peritoneal does not exclude the diagnosis of EPTB. Patients with a high clinical probability of pleural or peritoneal TB despite a negative Xpert MTB/RIF should be started on anti-TB treatment.

9.8. The current TB diagnostic algorithm in Ethiopia (Figure 1)

Rational use of TB diagnostics should consider the potential benefits of the test, cost and patient-centeredness. The Ethiopian national algorithm for TB diagnosis (last update in 2016), drug susceptibility testing and patient management follows the following recommendations:

- All individuals identified as presumptive TB case should have a bacteriological confirmatory examination either with Xpert MTB/RIF or sputum microscopy.
- Sputum microscopy is the preferred initial test for patients who are immunocompetent, aged above 14 years and have no prior TB treatment history.
- Xpert MTB/RIF is the preferred initial test for presumed/confirmed HIV positives, under 14 years children, presumptive DR-TB or EPTB patients.
- Any individual with previous TB treatment history and/or with confirmed/potential contact with DR-TB patients at household, workplace or congregated settings in the past 1-2 years should undergo drug resistance screening test at least for rifampicin using a rapid DST technique, preferably either by Xpert or first line-LPA.
- In patients in whom the diagnosis of TB remains in doubt despite negative results by bacteriologically confirmatory tests, additional clinical investigations may be done to get evidence supporting the clinical diagnosis.

9.9. Suggested new algorithm for TB diagnosis in Ethiopia

The current Ethiopian national algorithm is suggested for all TB suspects and ignores the specific Ethiopian TB epidemiology, where about 31% and 38% of cases notified have smear-negative PTB and EPTB, respectively. When new diagnostic tests are implemented, testing algorithms need to be modified. Modifications to algorithms are put in place only after a formal evaluation, review, and approval by officials within the Ministry of Health and the NTP.

Current WHO policy guidance recommends that Xpert MTB/RIF be used as an initial diagnostic test in individuals suspected of MDR or HIV-associated TB [11]. The guidance also provides a conditional recommendation that Xpert MTB/RIF may be used as a follow-on test to smear microscopy, especially in further testing of smear-negative specimens. Generalizing from adult data, the recommendation includes the use of Xpert MTB/RIF in children, acknowledging the difficulties in the microbiological diagnosis of childhood TB. The Xpert MTB/RIF test is also recommended for use with CSF, lymph nodes and other tissue samples.

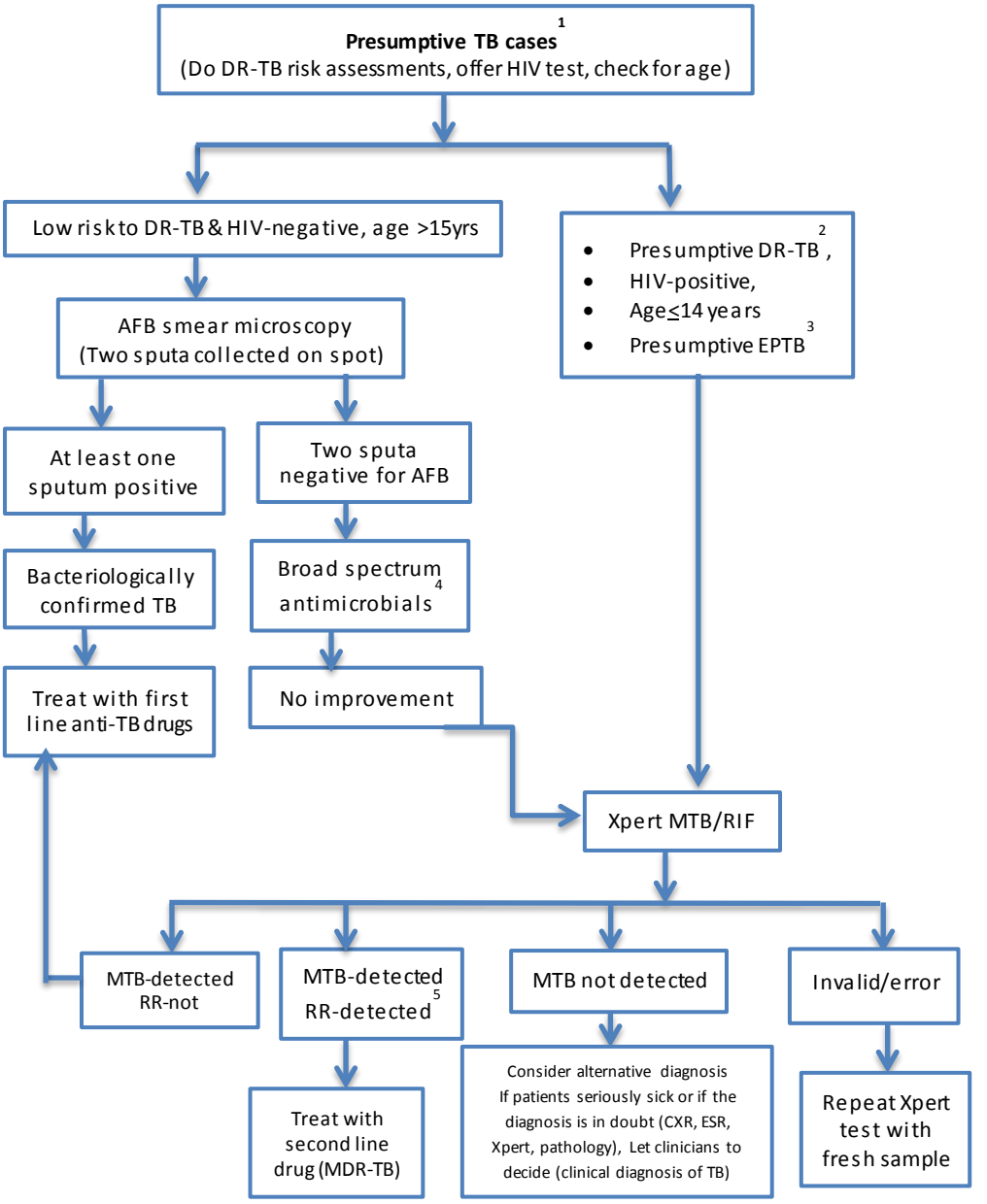
Despite these WHO recommendations on the use of Xpert MTB/RIF for further testing of smear-negative specimens, the current Ethiopian TB diagnostic algorithm recommends broad spectrum antimicrobial trial for smear-negative PTB patients, as an intermediate step. The existing national TB algorithm also recommends Xpert MTB/RIF as the initial diagnostic test for all forms of EPTB, although the test is not recommended for use with all types of extrapulmonary specimens. It is recommended for use with CSF, lymph nodes and other tissue samples. However, according to the WHO report/guideline, the test has low sensitivity for pleural and peritoneal fluids and data are limited for its sensitivity with stool, urine or blood specimens.

Based on our findings (Tadesse *et al*, 2016, and unpublished data in Chapter seven), WHO 2013 policy recommendations and expertise consultations, we suggest to change the Ethiopian algorithm as shown in **Figure 2 and 3.** We propose a separate detailed algorithm for extrapulmonary forms of TB considering the complex and multifaceted nature of the disease, difficulty in diagnosis and high prevalence in Ethiopia. Based on our new algorithm the following is recommended:

- Xpert MTB/RIF should be used as a follow-on test to smear microscopy, especially in further testing of smear-negative specimens. Only if the Xpert MTB/RIF test is negative, broad spectrum antimicrobial trails and further clinical evaluations should be undertaken.
- Xpert MTB/RIF should be used as the initial diagnostic test for lymph node specimens from patients suspected of having TB lymphadenitis.
- In areas where Xpert MTB/RIF test is not available, cytology followed by LED-fluorescence microscopy should be considered for patients suspected of having TB lymphadenitis, the former being a sensitive screening technique, the latter having a higher specificity.
- Xpert MTB/RIF should be used as the initial diagnostic test for cerebrospinal fluid (CSF) specimens from patients suspected of having TB meningitis given the severity of disease, rapidity of results and relatively good sensitivity compared to other tests.
- Xpert MTB/RIF should not be used as the initial diagnostic test for patients suspected of having pleural or peritoneal TB, given the very low sensitivity and the still relatively high cost of Xpert MTB/RIF. Clinical diagnosis, antibiotic trail, and clinical judgment for treatment decision remain an essential part of the diagnostic pathway until rapid and more sensitive tools are available.

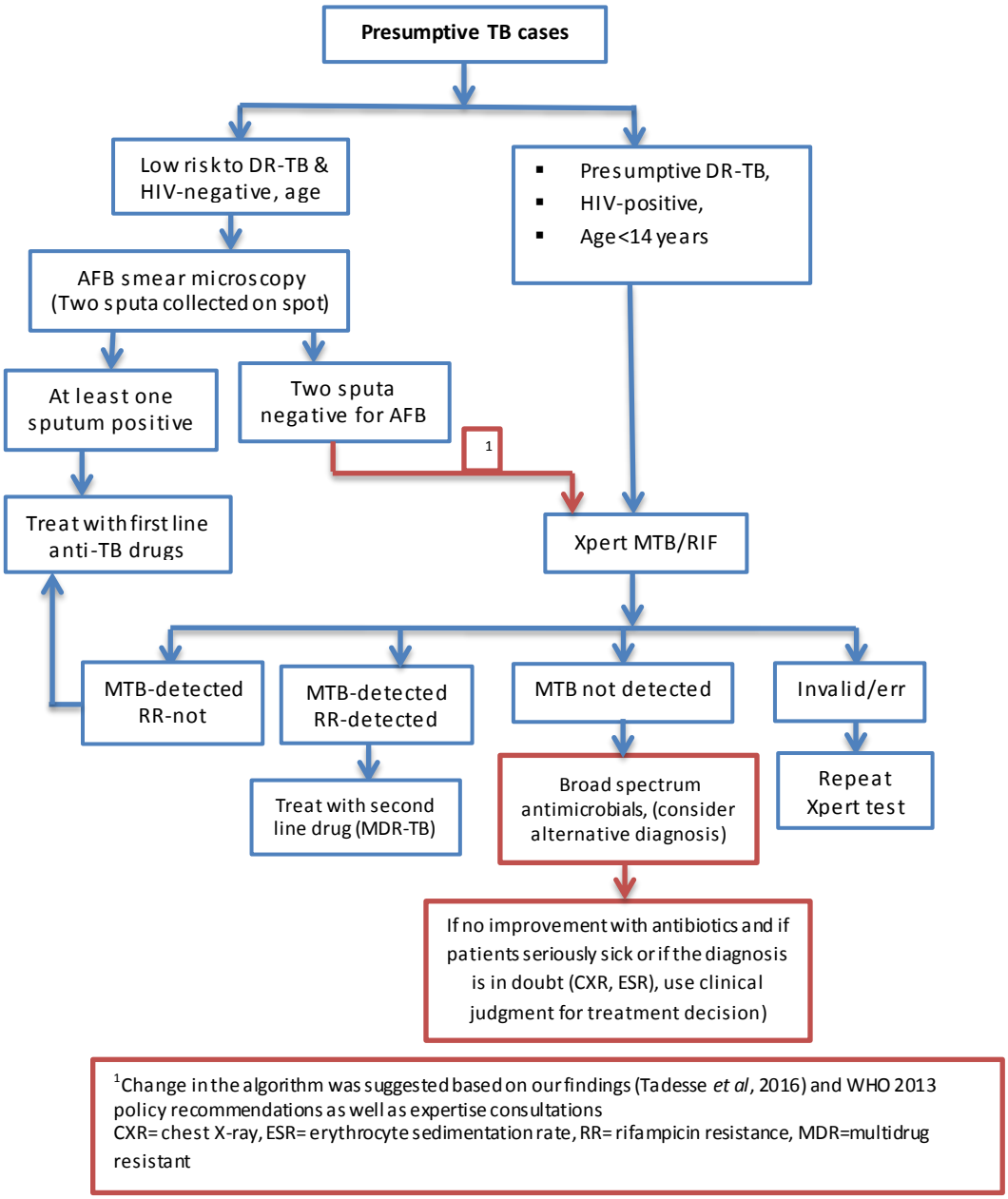
Overall, the usefulness of Xpert MTB/RIF to diagnose pleural or peritoneal TB is limited by its poor sensitivity. Other diagnostic approaches such as interferon gamma release assays (IGRAs) and adenosine deaminase could be integral part of the diagnostic algorithm for the diagnosis of hard to diagnose patients such as pleural or peritoneal TB [32]. In laboratories where culture is available, it could be considered as a follow-up test for smear- and Xpert MTB/RIF-negative patients though the incremental yield of culture over Xpert MTB/RIF is low in EPTB cases.

Figure 1: The existing TB diagnostic algorithm (NTP – MOH Ethiopia - 2016)



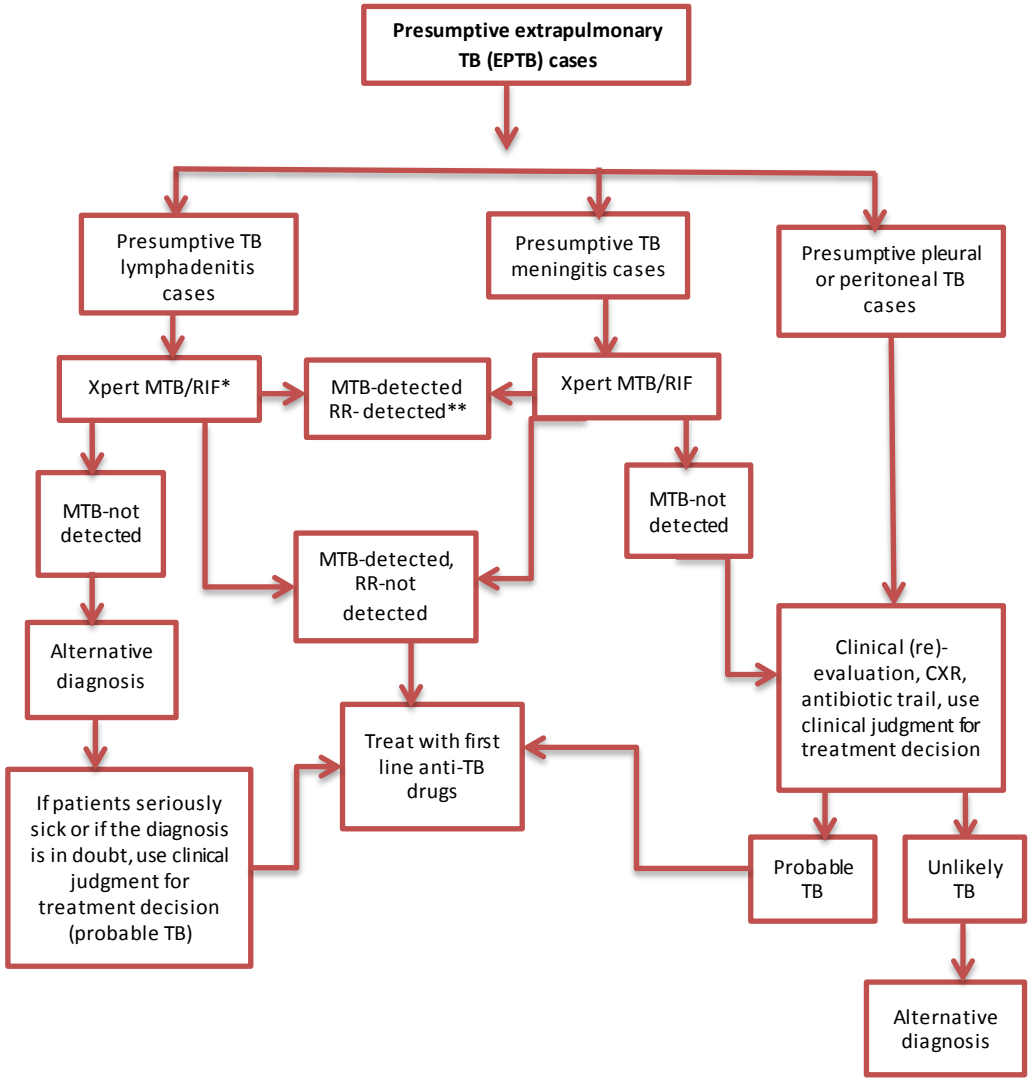
[1]Presumptive TB is defined by having symptoms & signs consistent with TB (cough≥2 weeks or any duration in HIV-positive), [2]Presumptive DR-TB: those patients with previous TB history, failures/late converters, or known contacts with DR-TB patients
[3]Liquid specimens (such as CSF) may be subjected to Xpert MTB/RIF without additional processing
[4]Broad spectrum antimicrobials (excluding fluoroquinolone or anti-TB drugs) is to be administered for 10-14 days, [5]RR-TB result in patients with low DR-TB risks needs to be re-confirmed with Xpert test on fresh specimen, -if result shows RR-TB again, treat with second line drugs, if not treat with first line and proceed with culture and DST

Figure 2: Suggested new algorithm for PTB diagnosis (proposed changes indicated in red box)



[1]Change in the algorithm was suggested based on our findings (Tadesse *et al*, 2016) and WHO 2013 policy recommendations as well as expertise consultations
CXR= chest X-ray, ESR= erythrocyte sedimentation rate, RR= rifampicin resistance, MDR=multidrug resistant

Figure 3: Suggested new algorithm for EPTB diagnosis (proposed changes indicated in red box)



[1]Any TB case with involvement of organs other than the lungs, e.g. lymph nodes, pleura, abdomen, genitourinary tract, skin, joints and bones, meninges

* If Xpert MTB/RIF test is not available cytology followed by LED-Fluorescence microscopy can be considered

**If rifampicin resistance is detected by Xpert MTB/RIF, patients should be referred to MDR-TB treatment center for second line anti-TB

9.10. MTBc lineages causing TBLN in Southwest Ethiopia

TB control strategies might significantly be affected by differences in virulence, epidemiologic characteristics and epidemiology of particular strains of the MTBc. Molecular epidemiology studies allow the identification of circulating strain types, understanding of transmission dynamics, as well as investigations of the evolution of the MTBc strains. In Ethiopia, few molecular epidemiological studies have been done so far mostly in the capital city, Addis Ababa and Northern Ethiopia [33, 34, 35].The causative agents of TBLN in Ethiopia are not systematically reported. Considering the high percentage of cervical TBLN recorded in Ethiopia (at least 20% of all reported TB cases), it is plausible that *M. bovis* might play a role. In chapter eight (**Paper VI**) of this thesis, we presented in-depth analysis of the population structure of MTBc strains causing TBLN in Southwest Ethiopia based on spoligotyping, 15-loci MIRU-VNTR typing and qPCR-SNP analysis.

We found that TBLN in Southwest Ethiopia was caused by a wide diversity of MTBc strains with predominance of the Ethiopian specific sub-lineages within Lineage 4 followed by Lineage 3 and Lineage 4- Haarlem. Our analysis also revealed the presence of the typical Ethiopian Lineage 7 in Southwest Ethiopia as well as Ethiopian specific families within the Lineage 4 also found to cause TBLN in Northern Ethiopia [31], where Lineage 3 (Delhi/CAS) is more common. Despite some minor difference in frequency, the overall MTBc strain distribution in Southwest Ethiopia is similar with distribution of strains from pulmonary and TBLN patients in different regions of Ethiopia [34, 36], suggesting that pathogen-specific genetic factors unlikely explain the high rate of TBLN in Southwest Ethiopia. Moreover, we observed high clustering rate for Ethiopia_3 isolates showing clonal similarity with isolates from North Ethiopia. This emphasizes the importance of strengthening laboratory diagnosis of TB including culture and intensified case finding to interrupt chains of transmission.

The overall contribution of *M. bovis* for TBLN in Southwest Ethiopia is minor. Our study reflected that TBLN in Southwest Ethiopia is caused by a variety of *M. tuberculosis* lineages, similar to the ones causing pulmonary TB, suggesting that in Southwest Ethiopia, cases of TBLN arise from the same source as pulmonary TB, rather than from an external zoonotic source. Nevertheless, in order to address the public health importance of bovine TB in Ethiopia, further study is needed on cases of abdominal TB and other forms of EPTB where the prevalence of bovine TB in cattle is supposed to be high.

Chapter ten: Concluding remarks and future perspectives

The rapid and accurate diagnosis of all forms of TB, followed by appropriate treatment, is the cornerstone of global TB control. However, it has been acknowledged that the TB laboratory capacity available worldwide is insufficient to address the diagnostic challenges related to EPTB, smear-negative and drug-resistant TB. There is a high-priority for determining the feasibility of using low-cost, rapid, easy to perform and interpret diagnostic methods for detection of paucibacillary and MDR-TB cases in resource constrained settings. Meanwhile, more emphasis and attention is required for optimal usage of currently available diagnostics to improve diagnosis of smear-negative and EPTB.

In this doctoral thesis, we evaluated different types of diagnostic methods (conventional smear microscopy, LED-fluorescence microscopy and Xpert MTB/RIF) coupled with different sample processing approaches for the diagnosis of smear-negative TB and different forms of EPTB in Ethiopia. Our findings concluded that routine use of conventional smear-microscopy (concentrated FNA) and LED-fluorescence microscopy can improve bacteriological diagnosis of TB lymphadenitis patients; however these methods cannot be a standalone diagnostic test. However Xpert MTB/RIF has the potential to revolutionize TB diagnostic capability for clinicians managing smear-negative and lymph node TB, and significantly reduce the usual lengthy pathway to diagnosis and treatment. Xpert MTB/RIF has better diagnostic performance than conventional diagnostic tools and substantially increases TB detection among culture-confirmed as well as clinically diagnosed TB cases, especially in TBLN.

Based on our findings, we recommend that Xpert MTB/RIF should be used as a replacement test for usual practice including conventional microscopy and cytology for testing lymph node specimens from patients suspected of having TBLN in Ethiopia. Even though a negative Xpert MTB/RIF does not rule-out TB in CSF, Xpert MTB/RIF could still be considered as the initial diagnostic test for diagnosis of TB meningitis as it has higher sensitivity and provides a more rapid diagnosis than the conventional diagnostic tools usually practiced in Ethiopia. Implementation of Xpert MTB/RIF will, however, require ongoing studies for specific extra-pulmonary specimen types where volumes and specimen preparation procedures have not been defined. Studies must also assess the impact of the use of Xpert MTB/RIF on time to TB diagnosis and clinical outcomes, thereby permitting cost–effectiveness analyses to be performed. Further and better-harmonized validation studies are needed on performance of

Xpert MTB/RIF in urine and stool specimens which are easier to obtain with standardized and microbiologically well-defined reference standards.

The findings of this thesis also confirm the fact that Xpert MTB/RIF has suboptimal sensitivity for some forms of EPTB. Individuals presumed to have EPTB or smear-negative TB but with Xpert MTB/RIF-negative result should undergo further diagnostic testing, and those with high clinical suspicion for TB should be treated with anti-TB. With limited finances, priority must be given to the development of technologies that will reach those not being served by current diagnostic provision. Next-generation molecular tests (such as Xpert MTB/RIF Ultra), interferon gamma release assays (IGRAs) and adenosine deaminase (ADA) may further improve the diagnosis of TB in hard to diagnose populations such as pleural TB patients. Feasibility and cost-effectiveness studies of implementing IGRA's in the Ethiopian setting are warranted, given the still high cost of IGRA tests and level of infrastructure required.

Due to cost and technical requirements, the role of Xpert MTB/RIF as a POC test in primary health centers in low-income countries may be questionable. There is a need for a rapid, accurate true POC diagnostic test that is affordable and can be readily implemented in TB endemic countries. The GeneXpert Omni (Cepheid, Sunnyvale, CA, USA) has the potential to become more a POC test in remote areas in resource limited settings with relatively low patient throughput. GeneXpert Omni is a small and portable tool that has a rechargeable battery to provide power for 4 hours. Tests based on the detection of mycobacterial lipoarabinomannan (LAM) antigen in urine have also emerged as potential POC tests for TB, albeit restricted to severely immunocompromised patients. WHO recommended the use of the LAM test for the diagnosis and screening of active TB in people living with HIV. Additional rigorous studies are needed to evaluate and confirm the utility of the LAM assay for the diagnosis of EPTB at peripheral laboratories where the test could be performed as a POC test.

For the first time (paper VI), the genetic diversity of circulating MTBc strains causing TB lymphadenitis in Southwest Ethiopia was described. It is widely believed that strain-to-strain variation can have important phenotypic consequences including virulence, transmissibility and response to treatment. Furthermore, it is also known that such variation will affect future diagnostics, drugs and vaccines, and will therefore impact on product design and patient management. We found that the high rate of TB lymphadenitis in Southwest Ethiopia was

caused by a wide diversity of *M. tuberculosis* lineages with predominance of the Ethiopian specific sublineages. Zoonotic transmission of *M. bovis* infection has been excluded as a major factor in TBLN in our setting. Nevertheless, further research is needed in pastoral areas where the prevalence of bovine TB in cattle is known to be high, in order to have a better answer about the public health importance of this zoonotic disease in Ethiopia.

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Curriculum Vitae

Mulualem Tadesse joined Addis Ababa University in Ethiopia for his undergraduate study (BSc) in Medical Laboratory Technology in 2004. He graduated with a BSc degree in 2007. Then he started to work at Jimma University, Department of Medical Laboratory Science as Graduate assistant I, II and Assistant Lecturer, where he was involved in assisting, teaching and supervising undergraduate medical students. In 2010, he joined Jimma University, Post graduate school to pursue his Masters in Clinical Microbiology. For his master's thesis, he joined the Mycobacteriology Research Center at Jimma University in 2011 and started working clinical and operational research mainly in the field of tuberculosis and HIV. After completing his postgraduate (MSc) study in 2013, he started to work as Lecturer at Department of Medical Laboratory Sciences and Pathology, and as researcher at Mycobacteriology Research Center. In 2014, he started his PhD at University of Antwerp and Institute of Tropical Medicine, Belgium through IUC-JU program. Since December 2016, he has been promoted to the rank of assistant professor. Mulualem has published more than 15 articles in peer reviewed international journals.

Degrees

Bachelor of Science in Medical Laboratory Technology	September 204 - August 2007
Master of Science in Clinical Microbiology	November 2010 - March 2013
PhD student in Biomedical science	Since October 2014

Posts held

Assistant Professor	Since December 2016
Biosafety Officer at Mycobacteriology Research Center	Since September 2015
Head of Microbiology course team	November 2013 –June 2014
Assistant lecturer and Lecturer	March 2013 - November 2016
Graduate assistant	November 2007- March 2013

Skill sets

- **Laboratory:** Skilled at detection, isolation, identification and drug-susceptibility testing of mycobacteria using classical microbiological and molecular biological tools
- **Research:** Skilled at the integration of classical microbiological, molecular, cytological and clinical data in the study of (drug-resistant) tuberculosis for diagnostic method evaluation to improve TB diagnosis. Proficient at writing proposals and publishing research papers in peer reviewed journals.
- **Education**: Effective at teaching and guiding bachelor, and master students at the level of

project development, study design and set up, laboratory experiments, study analyses and writing

In service trainings

- Research stay at Mycobacteriology Unit of Institute of Tropical Medicine (ITM) and practical training on Spoligotyping technique at ITM, Antwerp, Belgium, from June – August, 2014
- Short Course in Clinical Research and Evidence based Medicine, Institute of Tropical Medicine, Antwerp, Belgium, May 26- June 24, 2016.
- Fluorescein-diacetate (FDA) vital staining' at National Reference Laboratory, Rwanda Biomedical Center, Kigali, Rwanda, March 16-20, 2015.
- Training on Dangerous Good transport (Infectious substance & other biological material)-Meeting IATA's requirement, Organized by World Courier, Cotonou, Benin, May9-10, 2016.
- Short term training on ''Introduction to Clinical trial'' (VLIR-OUS), Jimma University, October, 2013.
- Training of trainers (TOT) on AFB smear microscopy using Ziehl-Neelsen stain and fluorescent microscope and external quality assurance (EQA), Adama, Ethiopia, 2013
- TOT courses on CD4, Chemistry & Hematology laboratory, Organized by CU-ICAP, EPHI, Ethiopia.

Participation in national and international scientific meetings

1. Participation and Oral presentation of research article at the 2nd Asian-African Congress of Mycobacteriology, 25-28 February, 2017, Isfahan, Iran.
2. Certificate of attending scientific conference-the 9th International AIDS society (IAS) Conference on HIV Science, Paris, France- 23-26 July 2017.
3. Certificate of attendance on the 10th European Congress on Tropical Medicine and International Health takes place in Antwerp, Belgium, 16/10/2017 - 20/10/2017.
4. Certificate of recognition for my best oral scientific paper presentation at the 20th Annual Conference and 50th years anniversary of Ethiopian Medical Laboratory Association, Addis Ababa, Ethiopia, May 9-10, 2015.
5. The best Tuberculosis Research Abstract presentation at the 11th TB Research Annual Conference, Dire Dawa, Ethiopia, March 20-22, 2016

6. Oral presentation of research article at the first International Conference on (Re-) Emerging Infectious Diseases (ICREID) organized by Africa CDC, Addis Ababa, Ethiopia- March 12-14, 2018.
7. Participation on conference "Infectious Diseases and Women Health" and poster presentation of research abstract at UNESCO-MARS 2016 on 28 & 29 November 2016 at Addis Ababa, Ethiopia .
8. Poster presentation of research paper at 10th Tuberculosis Research Annual Conference organized by TB Research Advisory Committee (TRAC): March 21-23,2015, Adama, Ethiopia.