

**Drug sensitivity patterns of *Mycobacterium tuberculosis* complex
species isolated from tuberculosis lymphadenitis patients in
north-western Ethiopia**



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Drug sensitivity pattern of *Mycobacterium tuberculosis* complex species isolated from lymphadenitis tuberculosis patients in north-west Ethiopia

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LIST OF ABBREVIATIONS

ALIPB	Aklilu Lemma Institute Of Pathobiology
AMK	Amikacin
AMA	Amplification Mixes A
AMB	Amplification Mixes B
BCG	Bacillus Calmette Guerin
CDC	Centre for Disease control
CAS	Central and Middle Eastern Asian
CDR	Case Detection Rate
CS	Cycloserine
DEN	Denaturation Solution
DNA	Deoxi ribonucleic acid
DR	Direct Repeat
DST	Drug Susceptibility Tests
CAS	East-African Indian
EMB	Ethambutol
ETH	Ethionamide
EFY	Ethiopian Fiscal Year
EPHI	Ethiopian Public Health Institute
EA	Euro-American
EPTB	Extra-Pulmonary TB
FHCSH	Felege Hiwot Comprehensive Specialized Hospital
FNA	Fine Needle Aspirate
GGH	Gabmby General Hospital
HYB	Hybridization Buffer
IO	Indo-Oceanic

INH	Isoniazid
KM	Kanamycin
LAM	Latin America Mediterranean
LVX	Levofloxacin
LJ	Lowenstein-Jensen
A-LYS	Lysis Buffer
MDGs	Millennium Development Goals
MoH	Ethiopian Ministry of Health
M LPA	Molecular Line Probe Assay
MDR-TB	Multidrug-Resistant TB
MTBC	Mycobacterium Tuberculosis complex
BACTEC MGIT 960	Mycobacterium Growth Indicator Tube
A-NB	Neutralisation Buffer
NTM	None Tuberculosis complex
OADC	Oleic acid, Albumin, Dextrose and Catalase
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
RMP	Rifampin
RIN	Rinse Solution
SIT	Spoligotype International Type
SM	Streptomycin
TBLN	TB Lymphadenitis
WHO	World Health Organization

ABSTRACT

Drug resistance threatens the National Tuberculosis Control Program in several countries of the world. The drug sensitivity patterns, especially among tuberculosis lymphadenitis (TBLN) patients, are not fully understood in northwestern Ethiopia. This study aimed to identify drug sensitivity patterns of *Mycobacterium tuberculosis* complex (MTBC) strains isolated from TB lymphadenitis (TBLN) patients and association of strain types in northwestern Ethiopia. Study design was convenient sampling. Drug sensitivity testing (DST) against first line drugs, including isoniazid (INH), streptomycin (SM), rifampicin (RMP) and ethambutol (EMB), was performed using *Mycobacterium* Growth Indicator Tube (BACTEC MGIT 960) system and GenoType MTBDRplus assay. The study showed TBLN patients to harbor drug-resistant and MDR-TB. Among 82 MTBC isolates, 71 (86.5%) were sensitive to all first-line drugs and only 1 (1.2%) was multi-drug resistant (MDR). Any resistance to INH, RMP, SM and EMB was 3(3.65%), 2 (2.43%), 8 (9.75%) and 1(1.20%), respectively. This study showed that TBLN patients harbored drug-resistant TB and MDR-TB in north-western. Euro-American was the dominant lineage identified from 52.4% (43/82) of the isolates and 2(2.4%) of the strains were *M. Bovis*. Although the prevalence of MDR-TB low, detection high resistance of first line drug, Isoniazid, is an indication of its possible spread in the study area. It requires to strengthen national control Program in its effort to prevent and control TB and a close drug resistance monitoring to keep it low and ultimately eliminate it.

Keywords: TB lymphadenitis, drug resistance, mycobacterium TB complex strains, BACTEC MGIT 960, GenoType MTBDRplus assay.

1. INTRODUCTION

1.1. Background

Tuberculosis is an ancient disease and the origin has been associated with the Neolithic demographic transition and emerged about 70 thousand years ago (Comas *et al.*, 2013). Tuberculosis is caused by *Mycobacterium tuberculosis* complex belongs to the class Actinobacteria and genus *Mycobacterium* (Kasper *et al.*, 2005). It is non-motile, non-sporulated, aerobic and acid fast bacteria (Kasper *et al.*, 2005). *Mycobacterium* are Mycobacterium complex, Mycobacterium leprae and non tuberculosis mycobacterium (NTM); Mycobacterium tuberculosis complex (MTBC) members are causative agents of human and animal tuberculosis. Species in this complex include *M. tuberculosis*, the major cause of human tuberculosis, *Mycobacterium bovis* (*M. bovis*), *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium microti* and *Mycobacterium pinnipedii* (Ryan and Ray, 2004).

Tuberculosis (TB) is most commonly transmitted by inhalation of infected droplet nuclei (the dried residue of larger respiratory droplets), which are discharged in the air when untreated TB patient coughs or sneezes. In the lungs they are phagocytised by alveolar macrophages, invade sub-epithelial layers of lung tissues and cause localized inflammation. Infiltration with immune cells and formation of lung tissue granulomas are hallmarks of TB pathology (Russell *et al.*, 2010). Consumption of unpasteurized milk and milk products from infected cows can also be considered as the usual mode of transmission of *M. bovis* from animals to humans (Bates, 1980, Harries and Dye, 2006).

There is currently no effective vaccine protecting humans against TB. *Bacillus Calmette Guerin* (BCG) vaccine has a documented protective effect against meningitis and disseminated TB in children. It significantly reduces the risk of TB by 50% (Colditz *et al.*, 1994). However, it no longer provides protective immunity in some populations (Behr, 2002).

TB is a major cause of morbidity and mortality worldwide. For instance, In 2014, an estimated 9.6 million people developed TB and 1.9 million died (WHO, 2015). In this connection, WHO have been targeting end TB strategy based on an assessment of TB epidemic and progress in TB diagnosis, treatment and prevention efforts. Even though , the progress of Millennium Development goal to halve TB Death and prevalence has not as expected , WHO end TB Strategy approved by the World Health Assembly in 2014 will targeted 90% reduction in TB death and an 80% reduction in the TB incidence rate by 2030, compared with 2015 report (Uplekar *et al.*, 2015).

Tuberculosis has also been one of the major causes of morbidity and mortality in Ethiopia as well. The population-based national TB prevalence survey in Ethiopia between 2010 and 2011 indicated that the prevalence of smear-positive TB was 108 per 100,000 population and that of bacteriological confirmed TB was 277 per 100, 000 adult population (Kebede *et al.*, 2014). TB incidence rate has been declining at an average rate of 3.9% per year since 1998 despite TB is remaining challenge to TB control program (EMoH, 2013). Ethiopia has been planning to reduce TB Prevalence , Incidence and Mortality rate by 35%,30% and 45% from the 2013 level by 2020 respectively (EMoH, 2013).

1.2. Extra pulmonary tuberculosis

The clinical manifestation of TB can be pulmonary or extrapulmonary (outside the lungs). The highly vascular areas like a lymph node, meninges, kidney, spine and growing end of bones are usually affected (Swaminathan and Narendran, 2008).

When a person inhales droplet nuclei containing *M. tuberculosis* that reach the alveoli of the lungs, alveolar macrophages ingest the bacilli and form granuloma and the majority of bacilli are destroyed or inhibited. When alveolar macrophages die, the remaining small amount of bacilli released inside and outside lung like regional lymph nodes, kidneys, brain, and bone (CDC, 2014). In this connection, *M. tuberculosis* enters the body and undergoes lymphatic dissemination. Tonsils are the most common routes of entry for the

pathogen. During the initial stage of infection, the lymph nodes are discrete. Afterward, the lymph nodes coalesce and break open due to pus formation. The wound so developed may not heal even for years (Kandola and Meena, 2014).

In the United States of America, in 2014, a total of 9,421 TB cases were reported. Among TB reported cases 1,938 (20.6%) were extrapulmonary (CDC, 2014). The high Extrapulmonary case was reported In Maine state which was 42.9% (CDC, 2014). A study conducted in Poland from the years 2002-2010, among 626,093 cases of TB, 62,251 (8.2%) cases are extrapulmonary tuberculosis (EPTB). The proportion of EPTB among all TB cases was higher in women than in men and was higher in people aged 0-19 years than in other age groups (Rowinska-Zakrzewska *et al.*, 2013). Other study conducted in Turkey showed that among the total of 766 TB patients were enrolled, with 102 (13.3%) cases were extrapulmonary (Gunal *et al.*, 2011). Moreover, 2015 WHO report showed that in South Africa among 318, 180 notified cases in 2014, 33,522 are extrapulmonary, the main reason being high HIV prevalence in the community . The technical capacity of trained health workers and unavailability of diagnostic tools have increased EPTB diagnosis in the community (WHO, 2015).

In developed countries, 10-15% of TB cases have extra-pulmonary involvement, but in patients from high-incidence countries, the rate is much higher. People who are HIV-positive and infected with TB develop EPTB much more frequently, about 50% of cases(WHO, 2015). This is indicative of the fact that immune compromised patients have more risk of acquiring EPTB cases and this affects the available diagnostics which is smear microscopy as the gold standard for TB prevention and control (Golden and Vikram, 2005).

In Ethiopia, from 2014/15, a total of 135,831 all forms of TB cases were reported with a TB case notification rate of 151 per 100,000 population. This report was higher than that observed in 2013/14 (133 per 100,000 population). Out of 135,831 cases, 29.8% were EPTB (MoH, 2014/15). Previous WHO report showed that TBLN is the most common

form of EPTB and accounts for 80% of all new EPTB cases in Ethiopia (WHO, 2011). This indicates that there is a high proportion of TB Lymphadenitis (TBLN) among EPTB cases diagnosed. The proportion is higher in Amhara region compared to other regions in the country (MoH, 2014/15).

1.3. TB Diagnostic Methods and Treatment

The signs and symptoms of TBLN is mainly slowly developing and painless enlargement of lymph nodes, followed by matting and eventual drainage of pus (MoH, 2008). Moreover, the symptoms of LNTB may include fever, weight loss, fatigue, and night sweats (Kandola and Meena, 2014). The diagnosis of EPTB mainly depended on clinical diagnosis as opposed to smear-positive pulmonary TB. This is due to limit diagnostic capacity, shortage of trained health workers, inavailability of diagnostic tools and facilities. As a result, there has been limited focus or attention given to the diagnostics of TB lymphadenitis and more specifically drug resistance among patients suffering from TBLN has been compromised (WHO, 2011).

According to the existing Ethiopian guideline for the diagnosis of TBLN, patients will start anti-TB treatment if fine needle aspirate (FNA) cytology is suggestive. This is compromised by lack of the facilities and trained health workers to do the test in most of the health facilities in the country, EPTB diagnosis has been compromised and the cases reported are definitely underestimated of the expected cases or less is detected of the cases that could be detected. In addition, low sensitivity and specificity might have led to inappropriate treatment and to a delay in the real diagnosis. So that, using culture methods as a gold standard despite a diagnosis of the required time to be long.

Recent advance and development of molecular techniques have improved the speeds, sensitivities, and specificities due to its short turnaround time and automation of the procedure like Genotype® MTBDRplus, Xpert MTB/RIF assay, reverse line blot hybridization Pyrosequencing, TB-Biochip oligonucleotide are mentioned. Ethiopia is one of the countries starting some molecular techniques like Line Probe Assay and Gene

expert in limited facilities(EMoH, 2013). However, sample transportation, capacity to take an adequate sample for testing and related factors affected its utilization despite the general agreement that using Gene Xpert for testing could increase the yield of the samples and facilitate the identification of RIF resistance patient.

1.3.1. Mycobacterium Growth Indicator Tube (BACTEC MGIT 960)

BACTEC MGIT 960 is a newly developed non-radiometric, fully automated, continuously monitoring system. The MGIT tube contains 7.0 ml of modified Middlebrook 7H9 broth base. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for growth of many mycobacteria, especially those belonging to *M.tuberculosis* complex. Addition of the MGIT PANTA™ is necessary to suppress contamination (Salman, 2006). In addition , the tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion (Salman, 2006).

Culture methods on Lowenstein-Jensen (LJ) and BACTEC MGIT 960 are currently used as gold standard despite that they take a long time (Kandola and Meena, 2014). Sharma *et.al* study showed that BACTEC MGIT 960 detected more cases as compared to solid culture (LJ media) with 12.3% additional sensitivity (Sharma *et al.*, 2014). The MGIT 960 system is an accurate method for DST of *M. tuberculosis* against isoniazid and streptomycin (Ahmad *et al.*, 2016). Moreover, BACTEC MGIT 960 takes shorter time to detection of TB (Cruciani *et al.*, 2004).

1.3.2. Line Probe Assay

Line Probe Assay is solid-phase hybridization Techniques (Piatek *et al.*, 2000). Based on the hybridization of amplified DNA from cultured strains or clinical samples, Currently, three line probe assays exist, the INNO-LiPA(R) the RMP. TB assay (Innogenetics, Ghent, Belgium) and the GenoType MTBDR assay (Hain LifeScience GmbH, Nehren, Germany) and second-generation Genotype® MTBDRplus (Hain LifeScience GmbH, Germany). *rpoB* gene codon 513 is a mutation responsible for RMP resistance and codon 315 of the *KatG* gene is a mutation responsible for INH resistance detected by all three assays. Genotype® MTBDR additionally detects *katG* mutations and Genotype® MTBDRplus detects both *katG* and *inhA* mutations (Piatek *et al.*, 2000).

The sensitivity and specificity of MTBDR_{plus} for INH resistance were 62.1% and 97.9% (Dorman *et al.*, 2012). The sensitivity of the MTBDR_{plus} assay was 87.5%, 100% and 95.2% for INH resistance, RMP resistance, and MDR respectively. The specificity was 100% for all resistance patterns. The dominating mutations in RMP and INH resistant isolates were in codon 531 of the *rpoB* gene and codon 315 of the *KatG* gene (Chryssanthou and Angeby, 2012)

1.3.3. Spoligotyping

Recent advances in molecular technology such as spoligotyping is a powerful tool to analyze the frequency of various *M. tuberculosis* genotypes and their transmission patterns, which can be used extensively for the development of effective infection-control policies and vaccine development. These genotyping methods are also used to identify the resistance strain by differentiating recurrence, re-infection and mixed infections with multiple strains of *M. tuberculosis* (Sankar *et al.*, 2013)

1.3.4. TB Treatment

TB treatment using different drug combinations is basically divided into two, first line and second line drugs. According to WHO recommendation Four-drug regimen INH,

RIF, pyrazinamide (PZA) and EMB for first line treatment and EMB-PZA kanamycin (KM) or amikacin (AMK)-levofloxacin (LVX) - Ethionamide (ETH) - Cycloserine (CS) for second line treatment (WHO, 2001). The regimens that are efficient in pulmonary tuberculosis (PTB) are also efficient in Extra Pulmonary Patients (WHO, 2001).

1.3.5. TB Drug Resistance Testing

TB is a curable and preventable disease. However, drug resistance poses a serious challenge to TB control. It is a major public health concern in several countries including Ethiopia. In particular, multidrug-resistant TB (MDR-TB) defined by resistance to at least INH and RMP (Streicher *et al.*, 2012). Globally, an estimated 3.5% of new cases and 20.5% of previously treated cases have MDR-TB (WHO, 2015). Ethiopia is also among the high-MDR-TB burden countries. The prevalence of MDR-TB is increasing at an alarming rate from a baseline rate of 1.6% among new TB cases in 2005 to the current level of 2.3% in 2014; similarly, the rate has increased from 11.8% to 17.8% among previously treated cases (WHO, 2015). In addition, for a better diagnosis, drug susceptibility tests (DST) for *Mycobacterium* species are not available as routine tests.

Advances in molecular biology and the availability of new information generated after sequencing the genome of *Mycobacterium tuberculosis* increased the knowledge of the mechanisms of resistance to the main anti-tuberculosis drugs. Understanding the mechanisms of drug resistance in tuberculosis has helped to improve techniques for rapid detection and treatment. RMP is an important first-line drug for the treatment of tuberculosis (Zhang and Yew, 2009). Rifampicin resistance which results primarily from single-nucleotide substitution mutations in a small region of *rpoB*, the gene encoding the β -subunit of the DNA-dependent RNA polymerase. It is essentiality of RNAP for transcription. Most bacteria possess a single DNA-dependent RNAP enzyme comprising a multisubunit $\alpha\beta\beta'\omega$ core that forms a 'crab claw-like' structure. The β and β' -subunits constitute the main components of each 'pincer' of the claw, forming a groove that accommodates the template DNA and provides a catalytic site for

phosphodiester bond formation, a secondary channel for incoming nucleotides, and a separate exit for the growing RNA transcript. In a tight complementary fit, RIF binds to the *rpoB*-encoded β -subunit, thereby inhibiting transcription. Structural analysis of the *Thermus aquaticus* RNAP has shown that the RIF binding site is located within the DNA/RNA channel, but not at the active site. Moreover, RIF-bound RNAP retains the ability to catalyses formation of the first phosphodiester bond in a nascent RNA transcript, suggesting that RIF does not inhibit catalysis. Instead, it seems that the drug obstructs the path of a growing RNA chain of two to three nucleotides in length: once transcriptional elongation is in full progress, RNAP is no longer vulnerable to RIF-mediated inhibition (Koch *et al.*, 2014)

Isoniazid (INH) is the most widely used first-line anti-tuberculosis drug. Since its discovery in 1952, INH has been the cornerstone of all effective regimens for the treatment of tuberculosis disease and latent infection. *Mycobacterium tuberculosis* is highly susceptible to INH (minimum inhibitory concentration [MIC] 0.02–0.2 $\mu\text{g}/\text{ml}$). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. The most commonly mutated drug resistance locus, in codon 315 of *katG*, which confers high-level resistance to isoniazid, was substituted in 74% (478/642) of Beijing isolates and in 30% (106/355) of Euro-American isolates. A new nonsense SNP in codon 668 also mediated resistance, consistent with the requirement of KatG for activation of the pro-drug. All (478) of the Beijing *katG* mutants encoded a p.Ser315Thr substitution, whereas 11% (12/106) of Euro-American isolates encoded 1 of 3 alternative substitutions in codon 315 (Casali *et al.*, 2014)

Biadglegne *et.al.* 2014, study show that among 225 extrapulmonary isolates, resistance to INH, RMP, SM, and EMB was found in 8 (3.6%), 4 (1.8%), 10 (4.4%), and 4 (1.8%) isolate, respectively. Of the 212 new TBLN cases, three (1.4%) were MDR-TB (Biadglegne *et al.*, 2014). A similar study by Ismael *et.al* indicated the highest rate of

drug resistance was reported to STP (10.2%) and INH (8.4%). The overall prevalence of drug resistance to at least a single drug is 77/230 (33.5%) (Esmael *et al.*, 2014).

A study conducted on a community-based survey in Ethiopia by Getahun *et.al*, 2015 reported among 90 confirmed and valid TB isolates MDR-TB was detected in 4.4 % of the isolates whilst the rest (60/90) were susceptible to all drugs. Moreover, the strains to the lineages showed that 74.7 % (68/91) belonged to Euro-American(EA) lineage, 18.6 % (17/91) to East Africa Indian (EAI)lineage and the remaining 6.5 % (6/91) belonged to Indo-oceanic (IO) lineage (Getahun *et al.*, 2015).

On the other hand, TB strain diversities also have related impact for TB control worldwide and a key factor in helping to identify sources of infection. Previous research that classified *M. tuberculosis* complex isolates into nine major spoligotyping-based families: *M.Africanum*, *M. Bovis*, East-African-Indian (EAI), Beijing, Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), a European family X, and a default family T (Sebban *et al.*, 2002). A study conducted in Addis Ababa in four hospitals by Korma *et.al* indicates among 65 known extrapulmonary isolates identified lineage groups were 69.5 % of the isolates were Euro-American lineage, 20.3 % East-African Indian (CAS) and 5.1 % Indo-Oceanic. The rest 5.1 % comprised of Beijing, East Asian and *M.Bovis* each having one isolate (Korma *et al.*, 2015). Another study conducted in northwest Ethiopia recently identified four newly defined clades of TB strains associated with active PTB in up to one-third of the patients, namely Ethiopia 1–3 and Ethiopia H37Rv-like strains (Tessema *et al.*, 2013). Moreover, Biadglegne *et.al* identified strains among 196 TBLN isolates , the majority of strains belonged to the Delhi/CAS lineage, followed by Ethiopia 1 - 3, H37Rv and Beijing genotype, respectively (Biadglegne *et al.*, 2015).

2. STATEMENT OF THE PROBLEM

It is known that the Millennium Development Goals (MDGs) had the aim to halve TB prevalence and death rates, to reach case detection rate (CDR) of 84 % by 2015. However, current evidence indicates that the decline in TB incidence is not as fast as expected in the global plan and very slow in the sub-Saharan African countries in particular (WHO, 2015).

In Ethiopia, TB is principally diagnosed passively through evaluation of persons presenting to health institutions with symptoms and/or signs suggestive of TB despite there is no standard recording and reporting system for contact investigation to monitor the performance. Mycobacterium species culture is not available as a routine TB diagnostic method despite culture based methods using Mycobacterium Growth Indicator Tube (BACTEC MGIT 960) and Lowenstein-Jensen (LJ) are using as a gold standard. Moreover, Poor diagnostic setup is the main challenge to diagnose EPTB in the resource-limited setting.

TB is treated using different combinations of anti-TB drugs like INH, SM, RMP and EMB as the first-line treatment, can reduce mortality rates. If the patients have got an effective, timely, and proper treatment they have a high chance of being cured. However, improper use of drugs leads to the development of drug resistance and multidrug resistance TB (MDR-TB). Thus, TB drug resistance pattern identification for *M. tuberculosis* is important for both therapeutic guidance and surveillance of drug resistance pattern (Van Klingeren *et al.*, 2007)..

There is limited evidence in Ethiopia about the drug resistance pattern among patients with TBLN and treatment of patients with such cases is empirical in almost all cases. Hence, knowing the drug sensitivity profile of *M. tuberculosis* isolates is useful to control the disease. This study provided information on drug susceptibility patterns in TBLN and association of strain diversities with drug resistance.

2.1. Hypothesis

Drug resistance against first line anti-TB drugs have developed in TBLN patients in north-western Ethiopia and it is associated with *Mycobacterium* spp strain types.

3. OBJECTIVES

3.1. General Objective

- To assess the drug sensitivity pattern and association of drug resistance with strain type of *Mycobacterium* spp Isolated from TBLN patients in north-western Ethiopia

3.2. Specific Objectives

- To determine the drug susceptibility profile of *Mycobacterium tuberculosis* complex strains isolated from TBLN patients using conventional and molecular methods
- To assess the association of drug susceptibility profile and strain types

4. MATERIALS AND METHODS

4.1. Study Setting

The study was conducted in Bahir Dar City and the surrounding districts of Amhara Region, north-western Ethiopia (Figure 1). The Amhara Region is one of the nine regions of Ethiopia with an estimated population of 17 million (ECSA, 2007). Eighty eight per cent of its population lives in the rural areas and accessibility of the health services is limited, as only half of the population resides within 2 hours walking distance from a public health facility (ECSA, 2007).

The study settings were Felege Hiwot Comprehensive Specialized Hospital (FHCSH) and Gabmby General Hospital (GGH), which are located in Bahir Dar City. FHCSH is serving the population of Bahir Dar City and remote areas of northwest Ethiopia. The

total population served by the hospital is estimated to be 12 million(MoH, 2008). In this connection, GGH a private hospital serving the population in the City and its surrounding districts.

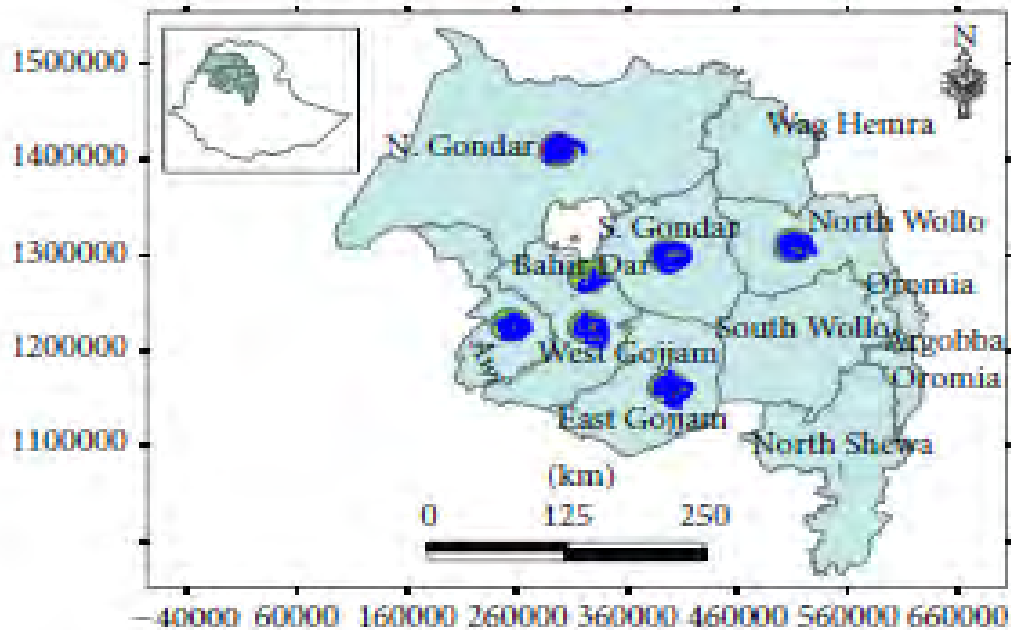


Figure 1: Amhara regional state and study site Source: (Nuru *et al.*, 2015)

4.2. Study Design

The design was a laboratory-based study on frozen samples stored following a cross sectional study

4.3. The study population

Samples collected from patients diagnosed as TB cases between September 2012 and January 2014 at FHCSH and GAMBY General Hospitals. After molecular epidemiological study (Nuru *et al.*, 2015) the samples were stored frozen at (20°C) at Aklilu Lemma Institute of Pathobiology (ALIPB), AAU, Addis Ababa.

4.3.1. Inclusion Criteria

- Isolates was the presence of correct isolates ID number , demographic data and also spoligotyping results.

4.3.2. Exclusion Criteria

- Insufficient volume for primary isolation and with visible contamination

4.3.3. Sample Size Determination and sampling technique

- Convenience sampling: this study was based on all samples available for the study purpose and the available samples were all tested.

4.3.4. Study Variables

Dependent Variables:

Drug resistance pattern of *Mycobacterium* isolates.

Independent Variables

Strain types and demographic characteristics

4.4. Specimen Collection

.Isolates were stored in ALIPB laboratory with temperature of 20°C, selected sufficient Isolates after prior communication and permission of Principal investigators and Ethics committee Approvals..

4.5. Conventional Drug Susceptibility Testing

A total of 96 *Mycobacterium* sp. isolates were available for this study, of which 82 BACTEC MGIT 960 positive isolates were examined for first line drug sensitivity testing using Mycobacterium Growth Indicator Tube (BACTEC MGIT 960) system. On MGIT tube 0.8 mL of the OADC enrichment was added followed by addition of 0.5 mL of stored *Mycobacterium* isolate sample. After inoculation, BACTEC MGIT 960 tubes were loaded into slots of the BACTEC MGIT 960 machine. The tubes were incubated until it flags positive or negative by its indicator lights, green or red color, respectively.

BACTEC MGIT 960 positive tubes were detected using the immunochromatographic method (SD Bioline TB Ag MPT64 Rapid test) to differentiate whether the isolate (formerly confirmed as M.TB) belongs to a *Mycobacterium* sp. complex or NTM. The test was performed by taking isolates from BACTEC MGIT 960 (liquid culture) and 80–100 µL of the specimen were dropped on the specimen placing area of the test plate of the device and after waiting 15 min, the reading area of the test plate were observed. Test results were interpreted within 60 min. a positive result indicate that the mycobacterium is MTBC but a negative result indicate the isolate is NTM (Mathewos *et al.*, 2015).

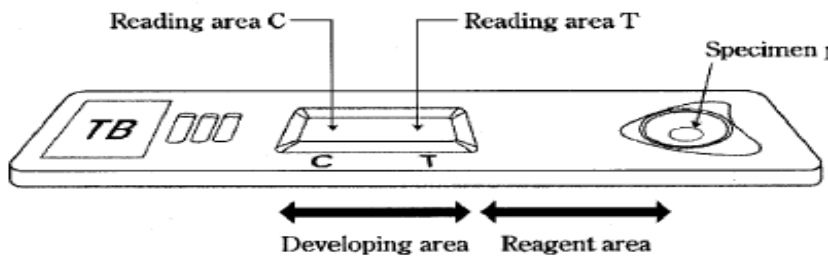


Figure 2: The schematic presentation of immunochromatographic.

MGIT cultures positive isolate at least 1 day but no more than 2 days directly conducted drug susceptibility test by reconstituted lyophilized antibiotics in distilled water and MGIT tubes supplemented with 0.8 ml of the enrichment solution (BACTECMGIT SIRE supplement; Becton Dickinson), the critical concentration for each drug was 5.0 µg/ml, 0.1 µg/ml, 1.0 µg/ml and 1.0 µg/ml for ETB,INH,RPM and STP respectively. On the other hand, if MGIT culture positive more than 2 days and above add 10ml and 4ml normal saline for control tube and drug test tubes respectively. All of the drug-containing tubes were inoculated with 0.5ml of MGIT culture. Then each drug was added according to the above mention concentration. The test tubes were incubated in the machine until the detector flashed on positive (resistant) or negative (susceptible) signs - green or red light, respectively. Finally, the interpretation of the results was done by comparing growth on control media and media containing drugs.

4.6. **GenoType MTBDR *plus* assay**

GenoType MTBDR*plus* assay was performed according to manufacturer's instructions (GenoType MTBDR*plus* product insert IFU-304A-02, Hain Lifescience, VER 2.0, 02/2012). The GenoType MTBDR*plus* is based on a molecular line probe assay (LPA) technology involving PCR amplification and binding of amplicons to specific oligonucleotide probes immobilized on a membrane strip. DNA extracted using Genolyse DNA extraction method (it is a chemical technique using Lysis Buffer (A-LYS) and Neutralisation Buffer (A-NB)). After DNA extraction, all reagents needed for amplification were included in the amplification mixes A and B (AM-A and AM-B). The 50 µl amplification mixture consisted of 10µl AM-A, 35µl AM-B, and 5µl DNA samples. A negative control sample which contains 5µl of distilled water instead of DNA solution is prepared for each run. Amplification was carried out in a Thermal Cycler (2720, Applied biosciences) and the PCR reaction was set at heating at 95°C for 15 min. This was followed by 10 cycles of denaturation at 95°C for 30 sec and annealing at 65°C for 2 min. This was again followed by another 20 cycles consisting of denaturation at 95°C for 25 sec, annealing at 50°C for 40 sec, and extension at 70°C for 40 sec, and final 1 cycle primer extension at 70°C for 8 min. Hybridization was performed with the TwinCubator (Hain Lifescience GmbH) and the procedure described in the manual enclosed in the test kit. Briefly, 20 µl of denaturation solution (DEN, blue) was pipette to each well of the tray to be used. Another 20 µl of amplified DNA sample was added to each well and mixed well by pipetting up and down several times and incubated for 5 min at room temperature. One ml of pre-warmed hybridization buffer (HYB, Sodium dodecyl sulfate) was added to each well and mixed until the solution had a homogenous color. After strips placed in each well, the tray was placed in a shaking TwinCubator (Hain Lifescience GmbH) and incubated for 30 min at 45°C. After the incubation time over, the HYB buffer (Sodium dodecyl sulfate) was aspirated completely with individual sterile pipettes, and 1 ml of stringent buffer (STR,) was added to each well and incubated for 15 min in a shaking Twin Curator at 45°C. The STR was removed completely and each strip was

washed with 1 ml rinse solution (RIN) for 1 minute on a shaking TwinCubator at room temperature. After the RIN was decanted completely, 1 ml of diluted conjugate was added to each well and incubated for 30 min on a shaking TwinCubator at room temperature. After conjugate was removed, each well was washed twice with 1 ml of RIN and once in 1 ml of distilled water with a shaking TwinCubator. After removing the water, 1 ml of diluted substrate was added per well and incubated at room temperature on TwinCubator under aluminium foil for a maximum of 10 minutes without shaking. The hybridization procedure was ended by rinsing twice with distilled water. The strips were removed and transferred to the GenoType MTBDRplus result sheet provided with the kit, and results were interpreted based on the presence and absence of bands. The *M. tuberculosis* isolate is considered RMP-susceptible if all of the wild-type S probes give a positive signal and all of the R probes react negatively. RMP resistance is indicated by the absence of one or more wild-type S probes. When RIF resistance is due to one of the four most frequently observed mutations, a positive reaction is obtained with one of the four R probes (Morgan *et al.*, 2005).

4.7. Molecular characterization of *Mycobacterium* isolates

Spoligotyping which includes three main steps; PCR amplification of specific spacer sequences of the genome, hybridization to a spoligomembrane, and detection was performed at ALIPB following the Standard Operating Procedure of Veterinary Laboratories Agency (VLA, 2004) of VLA- UK. It relied on a commercially available kit (Isogen Bioscience BVMaarssen, The Netherlands) for amplification of DNA from the DR locus, the region with the highest level of polymorphism in the *M. tuberculosis* chromosome (Kamerbeek, 1997). PCR amplification used 5 uL of extracted DNA from each sample mixed in PCR mix containing 12.5 uL Qiagen mastermix (MgCl₂, dNTPs, buffer, and Taq polymerase), 3.5uL sterile water and 2 uL of each primer (DRa: biotin-5_-CCG AGA GGG GAC GGA AAC-3_ and DRb: 5_-GGT TTT GGG TCT GAC GAC-3_) in a final volume of 25 uL. The amplification protocol required 15minutes at 96°C followed by 30 cycles of 1 minute at 96°C, 1 minute at 55°C, and 30 seconds at

72°C, with a final extension of 10 minutes at 72°C. The assay included two positive controls (chromosomal DNA from *M. tuberculosis* H37Rv and from *M. bovis* BCG P3) and a negative control (molecular grade H₂O). The amplified product was hybridized to a set of 43 immobilized oligonucleotides covalently linked to a nylon membrane (Isogen Bioscience BV, Maarssen, The Netherlands) at 60°C for 1 hour in a blotter with 45 lines (Miniblotter 45; Immunetics, Cambridge, Mass). Afterwards, hybridized DNA fragments were incubated with streptavidin conjugated to peroxidase (Boehringer Mannheim), and then detected by incubating for 1 minute in 20 mL of Enhanced chemiluminescence (ECL) detection reagent (Amersham, Buckinghamshire, England) and exposing to X-ray film for 5 minutes (Molhuizen *et al.*, 1998). The known strain formerly confirmed using Spoligotyping (Nuru *et al.*, 2015) was compared with drug profile, isolated samples identified by BACTEC MGIT 960.

4.8. Data Analysis

The data analysis was done using SPSS software version 20. Descriptive statistics was performed to get summary values.

4.9. Quality control

The sterility of the culture media was checked and 10% confirmed result were repeated. A known susceptible *M. tuberculosis* (H37Rv) control and resistant isolate which were tested by different methods was included in each test run of drug susceptibility testing.

4.10. Ethical Clearance

Ethical approval for this study were obtained Previously from ALEPB and Amhara regional state and for this study Addis Ababa University ,collage of Natural Sciences, Ethics committee was approved.

Since stored specimens collected from patients that had provided informed consent in an earlier study (Nuru *et al.*, 2015) were used in the study and it was not feasible to trace and

seek additional informed consent from the study participants, the study was conducted without the use of personal identifiers to maintain maximum confidentiality.

5. RESULTS

5.1. Demographic characteristics of the study participants

Among the study participants, 46.3% (38/82) were between the ages of 18 and 28 years, and male participants comprised 56.1% (46/82) of the TBLN cases. The majority of the patients with TBLN were new cases 90.2% (74/82) while the proportion of TBLN cases with the previous history of anti-TB treatment was 9.8% (8/82). The descriptive statistics on the demographic and clinical characteristics of the study participants is summarized in Table 1.

Table 1: Demographic and clinical characteristics of TB lymphadenitis patients in northwestern Ethiopia (n=82), 2016

Characteristics	Frequency	percentage
Age in years		
18-28	38	46.3
29-39	19	23.2
>40	25	30.5
Gender		
Female	36	43.9
Male	46	56.1
TB Category		
New cases	74	90.2
Re-treatment	8	9.8
Zone in Amhara region		
Awi-Agew	10	12.2
Bahir Dar city	3	3.7
East Gojam	16	19.5
North Gondar	1	1.2
North Wollo	2	2.4
South Gondar	29	35.5
West Gojam	21	25.6

5.2. Conventional and molecular drug sensitivity testing

Among 82 BACTEC MGIT 960 positive isolates, 86.5% (71/82) were sensitive to the four first-line drugs, namely INH, RMP, SM and EMB. Mono-resistance was highest for SM (6) followed by INH (2). Any Resistance to INH was detected in three cases (3.65%), of this one case was previously treated. Any SM resistance was detected in eight (9.75%) cases; of which two was previously treated. Any resistance to RMP was detected in 2.43% (2/82). Both INH and SM resistance was detected in 1.2% (1/82) cases. one case 1.2% was confirmed MDR case among new TB case.

The GenoType MTBDRplus assay was also performed on the 82 isolates and revealed 93% (77/82) of the isolates to be sensitive to INH and RMP. However, mono-drug resistance to INH and RMP were detected in 4.9% (4/82) of the cases for each. Similar to BACTEC MGIT 960, one MDR-TB case was also detected by GenoType MTBDRplus assay (Table 2).

Table 2: Drug sensitivity test of *Mycobacterium tuberculosis* complex strains isolated from TB lymphadenitis patients in northwestern Ethiopia (n= 82), 2016

	BACTEC MGIT 960			GenoType MTBDRplus assay		
	New patients	Re-treated patients	Total	New patients	Treated patients	Total
Total tested	74	8	82	74	8	82
Susceptible	66	5	71	71	5	77
Mono-resistance						
INH	1	1	2	1	1	2
RMP	1	0	1	1	1	2
SM	5	1	6	NS	NS	NS
Poly resistance						
MDR	1	0	1	1	0	1
INH+RMP	1	0	1	1	0	1
INH+SM+RMP	1	0	1	NS	NS	NS
INH +SM	1	0	1	NS	NS	NS
INH +EMB	0	1	1	NS	NS	NS
Any resistance						
INH	2	1	3	2	1	3
SM	6	2	8	NS	NS	NS
RMP	2	0	2	2	1	3
EMB	0	1	1	NS	NS	NS

INH= Isoniazid ; RMP= Rifampicin ; SM= Streptomycin ; EMB= Ethambutol ; MDR= Multidrug resistance; NS= None Specified

5.3. Strain typing and drug sensitivity profile

In the present study 32 spoligo patterns were identified. The overall diversity was 39%. Cluster strains were identified in 48.7%(40/82) isolates, and clustered strains were identified in SIT 289(14 isolates), SIT 3411(5), SIT 134(5), SIT 53(4), 3 isolates belonged to SIT149 and SIT25 each, and 2 isolates each belonged to SIT50, SIT168, and SIT952 . Among the isolates clustered, 47% (38/82) were pan-susceptible to all anti-TB drugs tested in both conventional and molecular tests, while 1.2% (1/82) was resistant to INH.

Table 5 shows the detection of 5 lineages from a total of 82 isolates isolated from TBLN patients in north-western Ethiopia. Out of these the Euro-American was the dominant lineage identified from 52.4% (43/82) of the isolates and 2(2.4%) of the strains were *M. Bovis*.

Further analysis of the data for association of strains with the drug profiles of lymphadenitis patients revealed one clustered strain of Euro-America lineage, belonging to SIT 3411, as resistant to INH (Table 6).

Table 3: The proportion of lineages identified from a total of 82 *Mycobacterium* sp. isolates identified from TB lymphadenitis patients in north-western Ethiopia, 2016

Type of lineage	Frequency	Percentage
Euro-American	43	52.4
East African Indian	28	34.2
Indo-Oceanic	4	4.9
<i>M. Africa mum</i>	5	6.1
<i>M. bovis</i>	2	2.4

Table 4: Association of Strains with the drug profiles of lymphadenitis patients in Amhara region, north-western Ethiopia, 2016

SIT	Octal number	Binary Number	Linage	Number of clustered isolates				
				Susceptible	Resistant			
					EMB	INH	RMP	SM
3411	777002377760771		EA	3	0	2	0	0
134	77777777720631		EA	5	0	0	0	0
149	777000377760771		EA	3	0	0	0	0
25	703777740003171		EAI	3	0	0	0	0
50	77777777720771		EA	2	0	0	0	0
53	77777777760771		EA	4	0	0	0	0
168	77777777720671		EA	2	0	0	0	0
289	703777740003571		EAI	14	0	0	0	0
952	603777740003771		EAI	2	0	0	0	0
Total				38	0	2	0	0

EA= Euro-America; EAI= East African Indian; INH= Isoniazid; RMP= Rifampicin; SM= Streptomycin; EMB= Ethambutol;

6. DISCUSSION

Most TB cases reported in this study were caused by *M. tuberculosis* and some caused by *M. Bovis*. This could be due to circulate *M. tuberculosis* in the community and could be due to the extrapulmonary manifestation of *M. tuberculosis* (Getahun *et al.*, 2015). Similar to the report of Biadlegne *et.al.*, (2015), *M. Bovis* among patients with lymphadenitis could be due to the circulating *M. Bovis* in the community, previous report indicated that, *M.Bovis* predominantly causes of TBLN (Cosivi *et al.*, 1998, Van Klingerren *et al.*, 2007). The Dominant lineage reported in the present study was Euro-American 52.4% (43/82), which is in line with previous studies in Ethiopia (Diriba *et al.*, 2013, Korma *et al.*, 2015).

The drug sensitivity pattern of patients diagnosed with TB lymphadenitis was reported in the present study using both conventional and molecular techniques. Resistance detected to STM (9.75%) was higher than that reported (4.4%) by Biadlegne *et.al.*, (2014) from the same geographic region (Northwest Ethiopia)(Biadlegne *et al.*, 2014). It might be due sample size and the method used, BacT/ALERT 3D system sensitivity rate to detect STM was 78%(Angeby *et al.*, 2003). Compared to earlier reports from different parts of Ethiopia (Abebe *et al.*, 2012, Tessema *et al.*, 2012, Yimer *et al.*, 2012, Getahun *et al.*, 2015), the present study indicated the proportion of patients with STM resistance to be in the lower range. This could be the impact of the TB control program in action, which includes the use of quality assured drugs for treatment, regular treatment supervision and the patients' adherence to treatment. Adherence to treatment and use of quality assured drugs contributes to high treatment success and reduces the possibility of developing drug-resistant TB. However, the possibility of inadequate supervision, adherence issues and quality of drugs could not be ruled out as causes for individual cases with resistance.

The overall prevalence of drug resistance determined in the present study, to at least one drug (14.6%), was similar to what (Tessema *et al.*, 2012) reported from the same part of

Ethiopia. This indicates the maintenance of sustained TB prevention and control efforts in the region, reducing the risk of intensification of drug resistance development.

On the other hand, the 3.65% rate of INH resistance detected among TBLN patients, in the present study, was higher than that previously reported (1.8%) from North West Ethiopia (Biadglegne *et al.*, 2014). The increase in the rate of INH resistance may have been due to a longer period of its use in the population, issues related to treatment adherence in the community, and the quality of drugs in addition to inadequate dose and inappropriate treatment and follow-up.

The detection of similar INH resistance rate by LPA and BACTEC MGIT 960 has indicated the usefulness of LPA as an anti-TB drug resistance surveillance tool in resource-constrained settings where there is limited availability and access to BACTEC MGIT 960. Furthermore, the finding of the present study is an additional proof that there is an increasing trend in INH resistance that could influence the performance of TB prevention and control efforts as INH is a potent first-line drug and plays a crucial role in the treatment of TB.

The level of MDR-TB recorded in this study (1.2%), is similar to the 1.3% reported previously from the same study area, North West Ethiopia (Biadglegne *et al.*, 2014), but lower due to study design compared to the general MDR (3.3%) among new cases reported by (WHO, 2015). This could be due to the same transmission pattern of MDR-TB from the lymph nodes of patients to others in the study area. In this study as the number of MDR cases detected was low, it was not possible to draw an association between the commonly known risk factors for MDR. However, since the commonly known factors, such as treatment failure, are valid in other MDR studies, it is possible that the same will have been true if the study sample size was larger.

Lack of published surveillance data in Ethiopia could be one of the reasons for lower reports on MDR as we do not have a national benchmark on the prevalence of drug resistance among patients with TB lymphadenitis. However, there are indicative reports

suggesting increases in drug resistance in the country, in general, more among new and re-treatment cases (National MDR survey, unpublished data).

The **high level** of drug resistance among new cases in this study might be due to the exposure of patients to drug-resistant strains of *M. tuberculosis* in the community and/or inappropriate and inadequate treatment (Abate *et al.*, 1998).

The fact that strain typing, which is used for predicting and monitoring drug resistance, revealed 48.7% of *M. tuberculosis* strains to be clustered, reinforces the suggestion that MDR-TB is probably a recent transmission in the present study as reported by Diriba *et.al.*, (2013).

7. CONCLUSION

1. The detection of all forms of drug resistance in the TBNL patients is an indication of its possible spread in the study area.
2. Although MDR-TB was low in the study area, the very fact that it existed is a cause for concern for a potential spread.
3. The high resistance to the first-line drug, Isoniazid, detected in the study may pose a challenge for the National control Program in its effort to prevent and control TB in Ethiopia.

8. LIMITATIONS OF THE STUDY

The main limitation of the study was the lack of detailed patient characteristics to identify more risk factors for drug resistance in the patients since it was conducted on stored samples from an earlier study. In addition, the fact that only one MDR case was detected limited further detailed statistical analysis of the data.

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10. DECLARATION

I, the undersigned, hereby declare that the work contained in this Thesis is my original work and that I have not previously in its entirety or in part submitted it at any university for a degree. All sources of material used for the thesis have been acknowledged.

Yohannes Sitotaw

M. Sc. Candidate

Signature

Date

Place: Addis Ababa, Ethiopia

11. ANNEXES

Annex 1 Protocol for BACET MIGT 960

Purpose

The **BACTEC MGIT 960** SIRE Kit is a 4 – 13 day qualitative test. The test is based on growth of the *Mycobacterium tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control). The **BACTEC MGIT 960** instrument continuously monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube is used by the instrument to determine susceptibility results.

Principle

The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base. The BACTEC MGIT 960 TB System has been found to boost culture positivity by 15-20% relative to conventional solid media and to substantially reduce the time to positivity. Liquid culture, however, is more prone to contamination. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for growth of many mycobacteria, especially those belonging to *M.tuberculosis* complex. Addition of the MGIT PANTA is necessary to suppress contamination.

Drug susceptibility testing can be performed by two MGIT tube are inoculated with the test culture. A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control). If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence. Growth is monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant.

Procedure

BACTEC MGIT 960 culture

1. Label BACTEC MGIT 960 tubes with isolate number.
2. Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA
3. Add up to 0.5 ml of a well mixed isolate to the appropriately labeled MGIT tube.
4. Incubate MGIT tube entered in the BACTEC MGIT 960 instrument after scanning each tube
5. Select Positive isolates for SD Bioline TB Ag MPT64 Rapid test

SD Bioline TB Ag MPT64 Rapid test

6. Label each cartridge with the sample identification number
7. Place 100µL of the prepared bacterial culture on the specimen placing area of the test cartridge. Pipette tips should be changed between samples
8. A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Line
9. The formation of a purple to red line on the reading areas labeled [T] and [C] of the cartridge indicates a POSITIVE result
10. The right section of the result window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Line
11. The formation of a purple to red line on the reading area labeled [C] of the cartridge but not [T] indicates a NEGATIVE result

12. If no line is observed on the reading area [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new cartridge
13. Examine the reading area of the test plate after 15 minutes

Drug Susceptibility test using BACTEC MGIT 960

14. Select a positive isolates and ready for DST (For the preparation of the test inoculum, a positive 7 mL **MGIT** tube should be used the day **after** it first becomes positive on the **BACTEC MGIT** 960 instrument (Day 1), up to and including the fifth day (Day 5) after instrument positivity.
15. The tube is a Day 1 or Day 2 positive, mix well and proceed to “Inoculation Procedure for Susceptibility Test.” If the tube is a Day 3, Day 4, or Day 5 positive, mix well then dilute 1 mL of positive broth in 4 mL of sterile saline (1:5 dilution). Use the diluted suspension for the inoculation procedures.
16. Label each MGIT tube with relevant drug and laboratory number and date
17. reconstituting lyophilized antibiotics in distilled water and MGIT tubes supplemented with 0.8 ml of the enrichment solution (BactecMGIT SIRE supplement; Becton Dickinson), the critical concentration for each drug was 5.0 µg/ml, 0.1 µg/ml, 1.0 µg/ml and 1.0 µg/ml for ethambutol, isoniazid, rifampicin and streptomycin respectively.
18. Add appropriate reconstituted drug solutions into each of the corresponding labeled BACTEC MGIT 960 tubes.

Drug	Concentration of Drug After Reconstitution*	Volume Added to MGIT Tubes for Test	Final Concentration in MGIT Tubes
MGIT STR	83 µg/mL	100 µL	1.0 µg/mL
MGIT INH	8.3 µg/mL	100 µL	0.1 µg/mL
MGIT RIF	83 µg/mL	100 µL	1.0 µg/mL
MGIT EMB	415 µg/mL	100 µL	5.0 µg/mL

* These drugs must be reconstituted using **4 mL** sterile distilled/deionized water to achieve concentrations indicated.

19. Aseptically pipet 0.1 mL of the organism suspension (see “Specimen Preparation”) into 10 mL of sterile saline to prepare the 1:100 Growth Control suspension. Mix the Growth Control suspension thoroughly.
20. Aseptically pipet 0.5 mL of the organism suspension (see “Specimen Preparation”) into each of the FOUR remaining drug tubes (STR, INH, RIF, EMB).
21. Inoculate 0.5 ml of this suspension into the growth control-labeled tube
22. Immediately recap the tube tightly and mix by inverting the tube several times.
23. Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
24. Be sure that the tubes are loaded according to the order specified for the AST set entry feature
25. Be sure that the caps are tightly closed
26. Open the desired MGIT 960 drawer and press the “tube enter” key.
27. The barcode scanner will light up
28. Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
29. Incubate MGIT tubes until the instrument flags them as positive
30. Check MGIT 960 daily for indicator lights flagging positive
31. Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer

Annex 2: Protocol GenoType MTBDRplus assay

Purpose

This procedure provides instructions for extraction, amplification and hybridization of mycobacterium isolates .

Principles

Extraction : DNA extraction is a procedure whereby DNA is obtained from bacterial cells or fragments by using molecular biology analysis, the Genolyse chemical method the bacterial cells in culture samples are chemically broken to expose the DNA by using a lyses buffer.

Amplification: Before amplification, amplification Mixes A (Taq polymerase, PCR buffer and nucleotides) and B(biotinylated primers,Mgcl₂ and are optimized for the PCR step of MTBDR_{plus} test. . The nucleotides acts as DNA precursors which will be used as building blocks during DNA polymerase (Hot Start *Taq*) elongate the DNA molecule.

Reveres Hybridization: The membrane strips used are pre-coated with specific probes complementary to the amplified nucleic acids. After chemical denaturing, the single amplicons bind to the probes. Thus, the probes reliably discriminates several sequence variations in the gene regions examined.. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a coloured precipitate

Procedures

Extraction:

1. Using a sterile disposable Pasteur pipette, transfer 1000µl of each thoroughly mixed liquid culture sample to labelled 1.5ml screw cap tube
2. Proceed similarly to the procedure for decontaminated smear positive sediments

3. Load the 1.5ml screw cap tubes in a micro-centrifuge with aerosol-tight rotor.
4. Centrifuge for 15 minutes at 10,000 RCF or 10263RPM
5. Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC
6. Discard supernatant from each tube by use of a 1000µl adjustable pipette
7. Resuspend each pellet in 100µl Lysis Buffer (A-LYS)
8. Mix the contents of each tube by use of a sterile tip followedby thorough vortexing for at least 15 to 20 seconds
9. Incubate the tubes for 5 minutes at 95 °C in a thermoblock
10. Add 100 µl Neutralisation Buffer (A-NB) and vortex the sample for 5 seconds
11. Load the tubes into a microcentrifuge and spin for 5 minutes at 13,000 x g
12. Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube
13. Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °C

Amplification and Detection :

1. Prepare a master mix containing AM-A and AM-B in a sterile screw cap tube (1.5ml) according to the number of samples and controls
2. Pipette 45 µl of the master mix to each labelled PCR tube
3. Add 5µl of DNA to corresponding mastermix PCR tubes in the PCR hood.
4. For first use, set up the thermal cycler to the correct amplification profiles according to the instruction manual (see SOP on Use and Maintenance of Thermal Cycler).
5. Run the specific program (**Ver.2-cul** for samples from solid or liquid culture)
6. After the cycles are complete, proceed to the detection stage. If detection cannot be performed on same day, store PCR tubes with amplicons at 4°C for a maximum of 7days
7. Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total). Pre-warm RIN (rinse solution) and sterile distilled water to room temperature

8. Pre-warm TwinCubator to 45°C
9. Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided pen according to detection worksheet.
10. Pipette 20µl DEN (denaturing solution) to one end of each well of a clean tray to be used
11. Add 20µl of corresponding amplified DNA sample to the denaturing solution in each well, and mix well by pipetting up and down 5 times
12. Incubate for 5 minutes at room temperature
13. Carefully add 1 ml HYB (hybridization solution) to each well and in the opposite end to the DEN/DNA mixture. Use single tip for each well. Do not splash mixture or contaminate neighbouring well
14. Gently tilt to shake and homogenize solution. Do not splash mixtures
15. Add each labelled strip to each well with coloured marker facing up. If strips turn over, re-position them with a fresh pipette tip. Strips must be completely covered by hybridization solution
16. Place tray on Twincubator and press “START” to incubate for 30 minutes at 45°C. From this point, press right arrow on Twincubator once to advance steps in protocol.
17. When alarm goes off, press right arrow key to stop
18. Remove HYB carefully by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change tips or Pasteur pipettes between wells
19. Wipe off condensation that forms on Twincubator lid before every incubation step.
20. Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in Twincubator at 45°C. Press right arrow key to start.
21. When alarm goes off, press right arrow key. Completely remove STR as previously described for HYB removal.

22. Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips for 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.
23. Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on Twincubator
24. When alarm goes off, press right arrow to stop
25. Completely aspirate CON-D solution using Pasteur pipette
26. Add 1ml RIN per well. Press right arrow and incubate for 1 minute on Twincubator
27. When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20
28. Remove RIN completely and wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator
29. When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.
30. Place on Twincubator under aluminum foil for a maximum of 10 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes
31. Wash twice for 1 minute with distilled water. Remove distilled water after each wash
32. Use forceps to transfer membrane strips to an absorbent paper and allow to air dry
33. Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water
34. Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol
35. Switch off the Twincubator after use

36. Use forceps to transfer strips to the GenoType MTBDR_{plus} Results Sheet provided with the kit
37. Align the bands Conjugate Control (CC) and Amplification Control (AC) on each strip with the respective lines on the sheet
38. Attach the strips to the results sheet using clear adhesive tape
39. Determine the band positivity and positions on each strip using the reference reading chart of the kit and mark the results on the worksheet
40. In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.
41. If a positive result is obtained with the negative control, the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
42. In order for patient results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that *M.tuberculosis* complex is present in the sample
43. If CC is negative the conjugation or substrate reaction was unsuccessful either due to error in the procedure or due to problems with the reagent
44. If AC is positive, errors during extraction and amplification set-up and presence of amplification inhibitors in the specimen can be excluded
45. Signal of AC can be weak or even absent while results for other bands (TUB, rpoB, katG and inhA locus controls) may be positive. This might be due to competitive reactions between AC and TUB, rpoB, katG, inhA during amplification. In this case, the strip can be evaluated.
46. A weak or missing AC band with negative test result for TUB, rpoB, katG and inhA locus controls may indicate potential mistakes during extraction and amplification set-up, or presence of amplification inhibitors. In this case, the test results are invalid
47. *rpoB* predicts RIF resistance, *katG* predicts high level INH resistance, *inhA* predicts low level INH resistance

48. The *rpoB*, *katG* and *inhA* each have a control band which must be present in order to interpret the results. Locus Control zones (*rpoB*, *katG*, *inhA*) detect a gene region specific for their respective genes. If the locus control zones are negative, then their respective mutation- specific positive bands cannot be considered for evaluation
49. A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster.
50. For results to be valid the bands (except CC) must be of intensity approximately equal to or greater than the intensity of the AC band.
51. If the TUB zone is negative, the tested bacteria does not belong to *M. tuberculosis* complex; therefore, presence or absence of any other bands (except CC and AC) cannot be considered for evaluation
52. When all wild type probes of a gene stain are positive and there is no detectable mutation within the examined regions, the tested strain may be considered sensitive for the respective antibiotic
53. In case of mutation, the respective amplicon cannot bind to the corresponding wild type capture probe on the strip due to the mismatch
54. The absence of a signal for at least one of the wild type probes may predict resistance to the respective antibiotic *indirectly*
55. Positive hybridization signal with a mutation-specific capture probe (for common mutations only!) may predict resistance to the respective antibiotic *directly*
Presence of rare mutations that do not have mutation-specific capture probes may only be indicated by the lack of hybridization with one or more wild type probes

Reference : Amita J, Vandana T, Guleria RS, Verma RK (2002). Qualitative Evaluation of Mycobacterial DNA Extraction, Protocols for Polymerase Chain Reaction. Molec Bio Tod 3:43-50

Ariefdjohan MW, Savaiano DA and Nakatsu CH (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutrition Journal* 2010, 9:23-31

GenoType® MTBDR*plus* VER 2.0. IFU-304A-01. Hain Lifescience GmbH.

Clinical Microbiology Procedures Handbook, 2nd edition, Henry D. Isenberg, Editor in Chief, 2004.

Annex 3: Protocol for Spoligotyping

The purpose of this protocol is to describe a method to detect and type bacteria of the *Mycobacterium tuberculosis* complex, including *Mycobacterium tuberculosis*, *Mycobacterium microti*, *Mycobacterium africanum* and *Mycobacterium bovis*. It also contains the method for stripping the sample from the membrane and makes it ready for reuse, and the method for spoligotyping of one membrane (i.e. 38 diagnostic samples + 5 controls). However, it is possible to hybridise two membranes simultaneously.

Spoligotyping

A PCR-based method is to simultaneously detect and type *Mycobacterium tuberculosis* complex bacteria

A. In vitro amplification of spacer DNA by PCR

Principle

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable them to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal *Mycobacterial* DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Löwenstein) can also serve as template. The PCR products are labelled with biotin, because primer DRa is biotinylated.

Procedure

1. Always include chromosomal DNA of *Mycobacterium tuberculosis* strain H37Rv and *Mycobacterium bovis*

BCG P3 as positive controls. Use water as a negative control.

2. Prepare the reaction mixture:

2 µl template DNA

3 µl primer DRa (0.2 µmol/µl)

3 µl primer DRb (0.2 µmol/µl)

20 µl 2×TaqPCR MasterMix

12 µl MQ water (to a final volume of 40 µl)

3. Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling:

3 min 94 °C 1 Cycle, 1 min 94 °C, 1 min 55 °C, 30 sec 72 °C 25 Cycles, 7 min 72 °C 1 Cycle, ∞ 4 °C

B. Hybridization with PCR product and detection

Hybridization of the biotin-labelled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

1. All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):

2×SSPE/0.1 % SDS, 42 °C,

2×SSPE/0.5 % SDS, 60 °C,

2×SSPE/0.5 % SDS, 42 °C.

2×SSPE, room temperature.

2. Add 25 μ l of the PCR products to 150 μ l 2 $\times$ SSPE/0.1 % SDS.
3. Heat-denature the diluted PCR product for 10 min at 100 $^{\circ}$ C and cool on ice immediately.
4. Wash the membrane for 5 min at 42 $^{\circ}$ C in 250 ml 2 $\times$ SSPE/0.1 % SDS.
5. Place the membrane and a support cushion into the miniblotted, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.
6. Remove residual fluid from the slots of the miniblotted by aspiration.
7. Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60 $^{\circ}$ C on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots.
8. Remove the samples from the miniblotted by aspiration and take the membrane from the miniblotted using forceps.
9. Wash the membrane twice in 250 ml 2 $\times$ SSPE/0.5 % SDS for 5 min at 60 $^{\circ}$ C.
10. Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.
11. Add 5 μ l streptavidin-peroxidase conjugate (500 U/ml) to 14 ml of 2 $\times$ SSPE/0.5 % SDS, and incubate the membrane in this solution for 60 min at 4 $^{\circ}$ C in the rolling bottle.
12. Wash the membrane twice in 250 ml of 2 $\times$ SSPE/0.5 % SDS for 10 min at 42 $^{\circ}$ C.
13. Rinse the membrane twice with 250 ml of 2 $\times$ SSPE for 5 min at room temperature.
14. For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 16 ml ECL detection liquid.

15. Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 20 min.
16. If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

C. Regeneration of the membrane

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times.

1. Wash the membrane twice by incubation in 1 % SDS at 80 °C for 30 min.
2. Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.
3. Store the membrane at 4 °C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

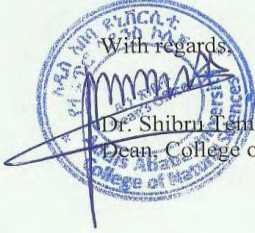
Annex 4 Ethics Approval Letters

COLLEGE OF NATURAL SCIENCES Addis Ababa University OFFICE OF THE DEAN የዲን ጽ/ቤት		የተፈጥሮ ሣይንስ ኮሌጅ አዲስ አበባ ዩኒቨርሲቲ Ref. ቁጥር CNSDO/183/08/2016 Date. ቀን January 14, 2016
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To Whom It may Concern

College of Natural Sciences Institutional Review Board (CNS-IRB) has reviewed an MSc. thesis project proposal entitled **“Drug Sensitivity Pattern of Mycobacterium Tuberculosis Complex Spices Isolated from Lymphadenitis Tuberculosis Patients in Bahirdar City and its surrounding, Northwest Ethiopia”** by Yohannes Sitotaw from the Department of Microbial, Cellular and Molecular Biology.

The proposal was approved for implementation.

With regards,

Dr. Shihru Temesgen
Dean, College of Natural Sciences

ፖስታል/Tel: +251-11-123 94 72 ፋክስ/Fax: +251- 11- 123 94 69	Please quote our reference number in your correspondence.	T.ሣ.ቁ/P.O.Box: 1176 Addis Ababa, Ethiopia ኢሜል/Email: dean_cns@aaau.edu.et
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“Examine all things; hold fast that which is good”

“ሁሉን መርምሩ. መልካሙን ያዙ”

Date: 10 December 2015

To: Ethiopian Public Health Institute
Addis Ababa

Subject: Consent for the use of stored sample for additional research work

This letter is referring to the student Yohhanis Sitotaw, to whom I have given my permission to conduct his MSc research work using my stored samples.

During my study period smear positive sputum and cytology positive fine needle aspiration (FNA) samples were collected from pulmonary and TB lymphadenitis patients who were diagnosed as TB cases between September 2012 and January 2013 at Felegehiwot Referral and GAMBY General Hospitals in Bahir Dar City. However my study and sample collection did not included children under the age of 18 years. Both sputum and FNA samples were cultured at the Bahir Dar Regional Health Research Laboratory Centre and following inactivation of AFB positive colonies the isolates were characterized for the different strains of mycobacterium TB complex species by spoligotyping. However few live isolates were freeze dried in freezing medium and kept for further analysis like MERU-VNTR and drug sensitivity test using classical and molecular techniques. These additional works were part of my PhD dissertation activity but because of time limitation and work burden I have negotiated and agreed with Yohannis Sitotaw to conduct the drug sensitivity test work for his MSc thesis.

I, therefore, kindly confirmed that the samples were collected from TB patients after ethical clearance obtained from Institutional Review Board of Aklilu Lemma Institute of Pathobiology (ALIPB) (**see the attached**), written permission letter from Amhara Regional State Health Bureau (**see the attached**) and oral go ahead permission from each study hospitals (after they have received the ethical clearance and support letters from ALIPB and Health Bureau, respectively). The consent given to the study subjects both in English and Amharic version is also presented with this letter.

With Regards



Anwar Nuru

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አክሊሌ ለማ ፓቶሎጂ መካነ ጥናት
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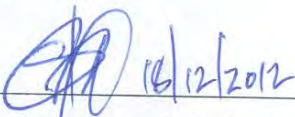
Ref. No: IRB/05-02/2013

Date: Dec. 18, 2012

To Whom It May Concern

Subject: Ethical clearance confirmation

The Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University in its meeting held on November 14, 2012 (minute Ref. No. IRB/02/2012/2013) and December 03, 2012 (minute Ref. No. IRB/05-02/2012/2013) has critically evaluated the scientific and ethical aspects of Dr. Anwar Nuru's PhD dissertation proposal entitled "Molecular epidemiology of *Mycobacterium tuberculosis* complex species and their transmission dynamics between farmers and their livestock in Bahir Dar city and its surrounding, north western Ethiopia". Thus, the board has endorsed the proposal for implementation.


Dr. Gobena Ameni, chair person

 18-12-12
Dr. Woldaregay Erku, secretary

Cc.

- Dr. Gobena Ameni, ALIPB
- Director's office, ALIPB

