ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

College of Natural and Computational Sciences,

Department of Microbial, Cellular and Molecular Biology

Mycobaterium Tuberculosis Infection in Northeastern Ethiopia: Studies on the Molecular Epidemiology, Drug Sensitivity Profile, Nutritional Status and Parasitosis Co-Infection as Major Risk Factors

By

Fikru Gashaw

A Dissertation Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology (Biomedical Sciences)

Addis Ababa, Ethiopia

July, 2020

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Dedication

This dissertation is dedicated to my lovely mother Fantaye Abayneh whose sudden death had broken my heart.

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Acronyms and Abbreviations

AAU: Addis Ababa University

AFB: Acid-Fast Bacilli

ALIPB: Aklilu Lemma Institute of Pathobiology

BCG: Bacillus Calmette-Guerin

BHDL: Biqat higher diagnostic laboratory

BMH: Boru Meda hospital

BMI: Body Mass Index

BTHC: Bati Town health centre

CHW: Community Health Workers

CMI: Cell-mediated Immunity

CRHC: Chefa Robit health center

DHC: Dessie health center

DNA: Deoxyribonucleic acid

DOT: Directly Observed Therapy

DRH: Dessie referral hospital

DRs: Direct Repeats

DST: Drug sensitivity test

E=EMB: Ethambutol

EMA: European Medicines Agency

EPHI: Ethiopian Public Health Institute

EPTB: Extra-pulmonary tuberculosis

FM: Fluorescent microscope

FMOH: Federal Ministry of Health of Ethiopia

FNA: Fine needle aspirate

GC: Growth control

GU: Growth unit

H=INH: Isoniazid

HEWs: Health Extension Workers

HIV: Human Immunodeficiency Virus

IFN-γ: Interferon Gamma

IGE: Immunoglobulin E

IL: interleukin

IPIs: Intestinal Parasitic Infections

JHC: Jaraniyo health center

KAP: Knowledge, Attitude and Practice

KHC: Kemise health center

LJ: Löwenstein Jensen

LPA: Line probe assay

M. tb: *Mycobacterium tuberculosis*

M/XDR: Multi or extensively drug resistance

MDR: Multidrug Resistant

ME: Molecular Epidemiology

MGIT: Mycobacteria Growth Indicator Tube

MGSS: Missed Grading Scale of the Sputum

MIC: Minimum Inhibitory Concentration

MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat

MLVA: multi-locus variable number tandem repeat analysis

MOP: Method of Proportion

MST: Minimum spanning tree

MTBC: *Mycobacterium tuberculosis* Complex

MUAC: Mid-upper Arm Circumferences

NTRL: National TB Reference Laboratory

PanACEA: Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics

PANTA: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin

PCR: Polymerase Chain Reaction

PGRS: pulsed-field gel electrophoresis

PTB: Pulmonary Tuberculosis

R=RIF: Rifampicin

RD: Regions of Difference

RFLP: Restriction Fragment Length Polymorphism

RNA: Ribonucleic acid

RR: Rifampicin Resistant

S=STM: Streptomycin

SAF: Sodium acetate-acetic acid-formalin solution

SIT: Spoligotype International Type

SNP: Single nucleotide polymorphism

Spoligotyping: Spacer Oligonucleotidetyping

SPSS: Statistical Package for the Social Sciences

STH: Soil transmitted helminths

TB: Tuberculosis

TBLN:TB lymphadenitis

TDR: Totally Drug Resistant

Th1: T-helper cell type 1

Th2: T-helper cell type 2

Tregs: T regulatory cells

XDR: Extensively Drug Resistances

Z = PZA: Pyrazinamide

Abstract

Background: *Ethiopia stands 12th among the 30 high TB burden countries with incidence rate of 165 cases per 100,000 population.Continuousand sustainable efforts should be exerted in different aspects including in research in order to reduce the burden of TB in the country.*

Objective*: The present study was conducted in northeastern Ethiopia to investigate the nutritional status of TB patients, TB co-infection with parasites, the types strains of M. tuberculosis circulating in the area and evaluate the drug sensitivity profiles the strains.*

Methods:*A cross-sectional study was conducted on 384 TB patients (286 smear positive pulmonary TB and 98 extra-pulmonary TB cases) who were visiting health care institution for seeking treatment. Anthropometric measurements were used for the assessment of the nutritional status of TB patients while direct wet mount microscopy, Kato-Katz and concentration techniques were used stool examination for parasite co-infections. Clinical examination, ZiehlNeelsen staining, mycobacterial culture, region of difference (RD) 9-based polymerase chain reaction (PCR), spoligotyping and mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing were used TB investigation. In addition, Mycobacteria Growth Indicator Tube (MGIT) test and GenoTypic MTBDRplus assay were used to evaluate drug sensitivity profiles of M. tuberculosis.Data were analyzed using descriptive statistics and Pearson chi-square.*

Results: *The prevalence of undernutrition was 58.6% using body mass index while it was 73.0% using mid-upper arm circumference. The overall prevalence of TB-parasitosis co-infection was 10.8%; intestinal helminths co-infection was 9.7% while intestinal protozoa co-infection was 1.9%. Clinically, TB lymphadentis accounted for 85.9% of the extra-pulmonary TB of which cervical lymphadenopathy was 75.3%. Culture positivity was confirmed in only 29.2% (112/384). Speciation of the isolates using RD9 PCR revealed 77.7% of the isolates were M. tuberculosis. But no signal was detected for the remaining 22.3% (25/112) isolates. Spoligotyping of 112 identified 92.9% (104/112) as interpretable spoligotyping patterns. Twenty-one percent of the isolates were grouped under 10 clustered strains while the remaining 79% (n=83) isolates were classified as singleton strains. On the other hand, 13.5% of the isolates were grouped under shared types and 86.5% were orphan. Furthermore, spoligotyping grouped 52.9%, 27.9% and 19.2% of the isolates in Euroamerican, Indio-oceanic and East African Indian lineages, respectively. DNA samples of 69 isolates were tested by 24-loci MIRU-VNTR typing and 56 had valid amplification products while the remaining 13 isolates had either incomplete or negative results. Each of the 56 isolates had distinct MIRU-VNTR profile and as a result, 56 different genotypes (strains) were detected MIRU-VNTR typing. The results of MGIT and MTBDRplus assay showed that 15.9% and 16.8% of the isolates developed resistance to either of the first line anti-TB drugs, respectively. The percentage of multi-drug resistance (MDR) M. tuberculosis was 8.4% (8/95) as detected by GenoTypic MTBDRplus assay. The agreement between MGIT and MTBDRplus assay in detecting resistance to Isoniazid (INH) was substantial (k=0.77) while it was near perfect in detecting either resistance to Rifampicin (RIF) (k=0.93) or in detecting MDR M. tuberculosis (k=0.90).*

Conclusion*: Undernutrition and co-infection with parasites were common in TB patients in northeastern Ethiopia. The strains M. tuberculosis circulating in northeastern Ethiopia were highly diverse and a significant proportion of the strains have developed drug resistance. Therefore, improvement of the nutrition and regular de-worming of the population in the area would contribute significantly to reduction of the burden TB and improves the response to TB treatment. Additionally, public education is required on the proper use of anti-TB drugs. Furthermore, special attention should be given to MDR TB cases so that their further transmission is contained.*

Key words: Mycobacterium tuberculosis, Molecular typing, Drug sensitivity, Parasite co-infection, Undernutrition, Northeastern Ethiopia.

1. Introduction

Tuberculosis (TB) causes major public health problems and developmental challenges of the world. Despite advances made in understanding its pathogenesis and management, *Mycobacterium tuberculosis* (*M. tb*) infects about a quarter of the global human populations. Of these, about 5-15% develops the disease during their life time and others remain as asymptomatic (WHO, 2019).

Morphologically, the *Mycobacterium* is identified as slender, straight or slightly curved, rod in shape existing as a single cell or in a thread like form. *M. tb* is a slow growing, obligate aerobe, non- encapsulated, non- spore forming, non- motile, acid-fast gram-positive with lipid rich cell wall (Glickman and Jakobs, 2001; Mathema *et al*., 2006). It mainly attacks lungs (pulmonary TB – 85%) and other body sites as an extra-pulmonary TB in less proportion (Charati and Moradi, 2013).

Although tuberculosis case detection is rising and the number of deaths due to the disease fell by 37% between 2000 and 2016, TB still remains as one of the top 10 causes of mortality from a single infectious agent. There is no country free of TB; but few countries have eliminated the disease as a public health threat. Its infection skewed heavily from economically well developed countries towards low-income and emerging economies. Most of the cases in 2018 were in the WHO regions of South-East Asia, Africa and the Western Pacific with a proportion of 44, 24 and 18%, respectively. Overall, about 87% of the world cases were from the 30 high TB burden countries; and two third of these were from India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (6%), Nigeria (4%), Bangladesh (4%) and South Africa (3%)(WHO, 2019).

Treatment of the disease by discovery of streptomycin was first introduced in 1944. Resistance was recognized by Pyle in 1947 against the drug and soon after its introduction as effective anti-TB chemotherapy. This leads to invention of another new TB drug (isoniazid), its initial clinical trials was made in 1952 that increased the effectiveness of chemotherapy toward TB. Consistently, TB was effectively treated with combinations of drugs streptomycin (STM), isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinaminamide (PZA). At the time, it was effective and seemed that the world's TB challenge was over with no more threat from the disease. However, resistance to all forms of the drugs emerged at high frequency when the drugs were used alone. These drug resistant bacteria resulted from spontaneous and random mutations of the bacterial chromosome either during a course of first-line TB treatment or due to the transmission of a drug resistant strains that leads to reduced susceptibility against specific agents and greater incidence globally. Beside the threat of this drug resistance, the risk of developing TB is enhanced due to co-infections and other immune profile altering factors including malnutritions (Daniel, 2006; Somasundaram *et al*., 2014).

Ethiopia belongs to one of the top 14 world’s high TB, MDR-TB and TB/HIV burden countries. Against this fact and major consideration given to TB in the country, a rapid molecular diagnostic automated test and molecular characterization of the bacteria is very limited. This limited information of genotypic characterization on the *Mycobacterium* strainsin the country could be due to scarcity of the diagnostic tools. The characterization is fundamental to determine the source of causative agent crucial for proper prevention and control program of tuberculosis. In fact, the country played a key role for the successful achievement of the global 2015 millennium development goal to stop TB with realistic implementation of the DOTs program since 1994 that leads to the targeted end TB strategy in 2035. Most of the molecular characterization technologies used in Ethiopia are region of differentiation and spoligotyping while other forms of characterization are done outside the country. Interestingly, the MIRU-VNTR technology was established for the first time in the country and applied in this study at Aklilu Lemma Institute of Pathobiology (ALIPB).

On the other hand, despite great concern given and increase of TB diagnostic health institutes in recent years, drug sensitivity testing is still challenging and not done on the spot in most health institutes in the country due to scarcity and lack of the diagnostic tools. Thus, the status of drug resistant cases was not clearly known against the TB control and treatment program. It is only at some hospital levels that the WHO recommendation of GeneXpert diagnostic test is applied for the detection of RR cases. However, due to limitations of cartridges, reagents, and other factors all *M. tb* infected cases were not tested by this Xpert technique. Instead the usual microscopic diagnostic method is used which cannot detect drug sensitivity profiles. The gold standard culture technique is also rare and not done at the health institute level with an exception of some regional, institutional and a single national laboratory found in the country.

It was estimated that 3.5 billion of the global population are infected with intestinal parasites of which 450 million are ill as a result of the infection (Stepek *et al*., 2006). These parasites are heterogeneous group of protozoa and helminths that live part or all of their lives in the hosts where they derive necessary nutrients, grow, and reproduce (Brooker *et al*., 2009, Keiser *et al*., 2010). The parasites interfere with nutritional and immune profiles of an individual promoting secondary infection, such as *M. tb*. Prevalence of intestinal parasitic infection is remarkably high in sub-Saharan African countries where TB is also common (Haque, 2007).

Awareness creation towards the disease is fundamental to generate better prevention and control programs of tuberculosis. Concerning this, the status of awareness is not well known in the country and only few assessments were conducted on knowledge, attitude and preventive practice towards tuberculosis in Ethiopia. Thus, molecular characterization, drug sensitivity profiles,nutritional status and TB parasitosis co-infections as major risk factors were the main objective in this study.

2. Review of Related Literature

2.1. Molecular Epidemiology of Tuberculosis

2.1.1. Tuberculosis and its causative agent

Tuberculosis is in the big group of the leading bacterial infectious diseases that cause profound mortality and morbidity throughout the world. Its pathogenicity could be pulmonary affecting lungs or extra-pulmonary which affect the central nervous system, gastro-urinary system, lymphatic system, circulatory system, bones, joints and skin. The extra-pulmonary TBs spread to these sites of infection through lymphatic or hematogenousdissemination. Tuberculosis might also be a combination of the two in which pulmonary TB has a symptom of cough, fever, chills, night sweats, appetite loss, weight loss, and easy fatigability. In addition to these clinical symptoms, the specific symptoms of extra-pulmonary tuberculosis depend on the site of infection (Tortoli *et al.*, 2012; Robert and Wani, 2013).

The continuous decline of TB in the early 1980s promised most medical experts as the disease would be completely eliminated in industrialized nations by the year 2010. However, at the mid of 1980s, the number of cases began to rise at an alarming rate specifically between 1985 and 1991. These sudden incidences of TB became common in developing countries which might be due to high correlation between HIV and TB. HIV infection suppresses the immune profile of an individual, emergence of drug resistance TB due to interruption of drugs when the patients feel healthier and other factors which might include immigration, poverty and undernutrition are the contributing factors (Barnes *et al*., 2011).

Everyone is liable to the disease with some groups as more susceptible for its activation. According to Pareek, *et al.* (2012), recent immigrants and refugees from countries with a high incidence of TB and those in close contact with a case of active TB including health professional are at greater risk. Furthermore, immuno-suppressed patients due to other infections like HIV and diabetics are liable to the disease. People living in substandard overcrowded conditions, undernourished, institutionalized groups including prisoners, aging and those who are dependent on drugs and alcohols are also at risk.

Phylogeny of *Mycobacterium tuberculosis* complex

Tuberculosis primarily caused by *M. tb* is undeniably the most successful terrible acid-fast bacillus with other closely related MTBC in rare cases. The less likely tuberculosis causing MTBC includes *M. bovis* which is responsible for bovine tuberculosis and include the vaccine strain *M. bovis* BCG; *M. africanum*, the main causative agent of TB in west Africa; *M. canettii*, a rare MTBC strain that produces smooth and glossy colonies; *M. microti*, which is a pathogen of voles and rarely infects humans; *M. orygis* and *M. caprae*, primarily isolated from goats; *M. pinnipedii*, also known as the seal bacillus; *M. suricattae* and *M. mungi* (Alexander *et al*., 2010).

The origin and global spread of human TB was expected as started from Africa. This assumption was supported by multi-locus sequence data from 108 global MTBC strains that hypothesizes human MTBC as originated in Africa and spreads out to other continents with the migrations of human population(Hershberg *et al*., 2008). Most of the MTBC lineages in Africa spread into Europe and Asia which later expand to others through the waves of human exploration, trade and occupation with some two or three phylogenetically 'ancient' lineages as staying in Africa (Comas *et al*., 2013).

Additionally, a new group of MTBC different from any known human-adapted complexes has been identified from Peru. It was closely related to contemporary *M. pinnipedii* which is adapted to seals and sea lions implying that marine mammals could have played a role in spreading TB from Africa across the Atlantic Ocean to the New World and transmitting to pre-Columbian human populations (Bos *et al.*, 2014).

A study of 20 variable RDs generated from insertion/deletion events were analyzed among 100 strains of the *M. tuberculosis* complex including *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. microti*, and *M. bovis*. Its finding showed the common progenitor strains as defined by the presence or absence of a *M. tuberculosis* specific deletion called "TbD1". This region was used to divide the MTBC as ancestral (TbD1 is present) and modern (TbD1 absent) strains. The modern strains include major global clades (Beijing and Haarlem). Deletion of RD9 and other subsequent deletions occurred in *M. africanum*, *M. microti*, and *M. bovis* that occurred before their divergence from *M. tuberculosis* and before alterations in TbD1 occurred. The genomes of *M. canettii* that have TbD1+ (ancestral) were the most intact and had not undergone as much genetic reduction through loss of RD loci and appeared to be closest to the most recent common ancestor for the *M. tuberculosis* complex (Brosch *et al.*, 2002). Other lineages were expected as evolving from the ancestral smooth coated *M. canettii* (Figure 1)

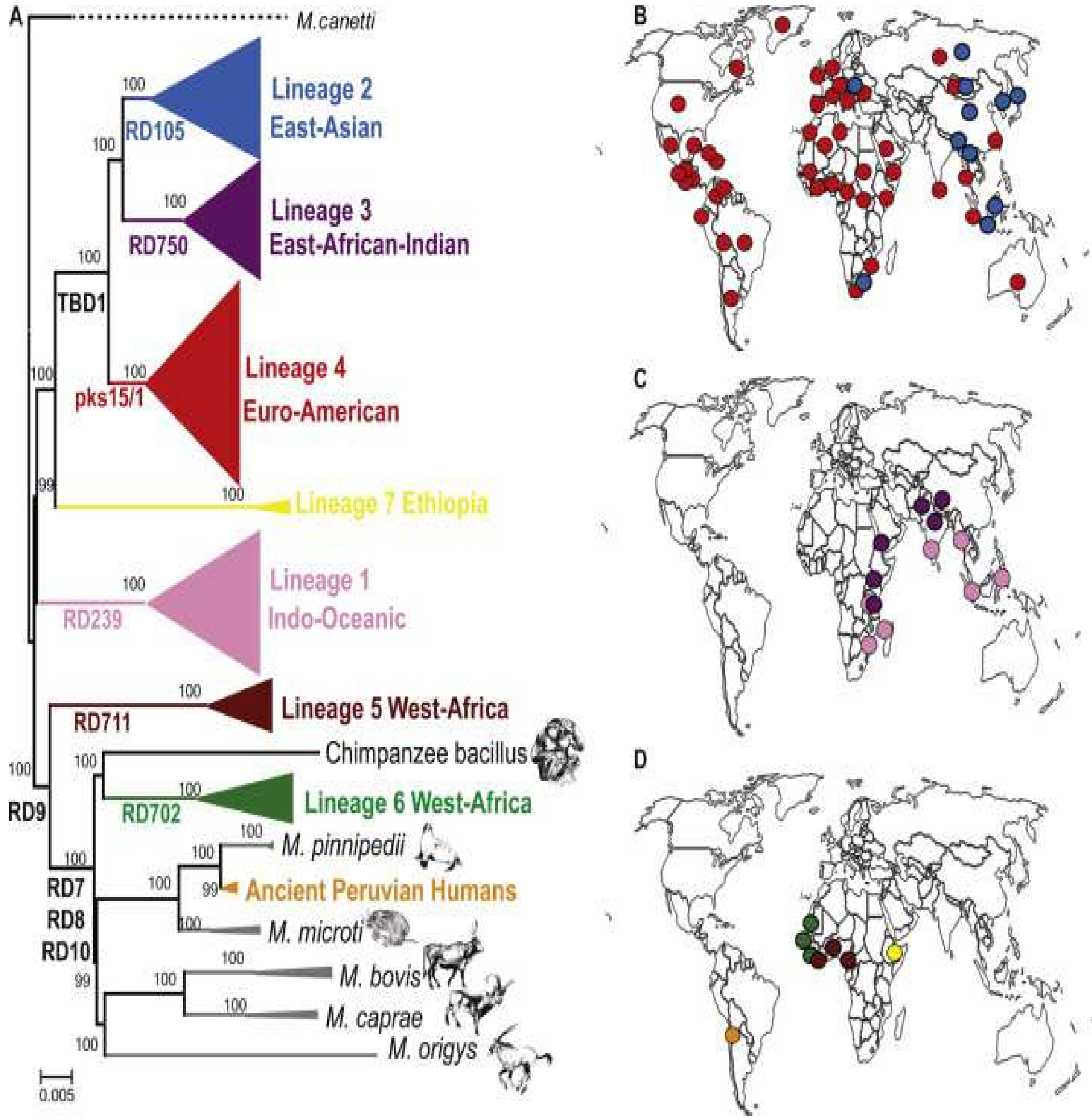


Figure 1. Global distribution of human-adapted M. tuberculosis complex

(a) The most likelihood phylogeny modified from. (b) The most geographically widespread lineages. (c) The intermediately distributed lineages. (d)The most geographically restricted lineages (Bos*et al.*, 2014).

2.1.2. Tuberculosis and socioeconomic burden

Although great attention and progress has been made on the control of TB, its global threat is still continuing. Higher prevalence of the bacterial infections is within the active working age group of 15 to 54 years inflicting negative impact on global socioeconomic prosperity (Jasmar, *et al*., 2002; Muniyandi *et al*., 2006). These economically productive age groups are also family forming with great social interaction in the community to enhance their ability and break free of poverty. Moreover, studies suggest that TB patients, on the average, lose 3-4 months of work time, resulting in lost wages. In the case of drug-resistant TB, it could take as long as two years and even more for treatment causing a significantly longer absenteeism from their work or loss of employment, social isolation, and long-term socioeconomic and psychological effects. Death of these income generating high energetic individuals due to TB increase the risk and things become harsh for their families (Morris *et al*., 2013).

Tuberculosis burden follows a strong socio-economic gradient between countries, within countries and within communities, and the poorest are those at greatest risk. It was an estimated US$ 13 billion annually for universal access to TB diagnosis, treatment and care with additional minimum estimates of US$ 2 billion for TB research by 2022. Although there is an estimate of a funding gap close to US$ 5 billion annually, the funding reached US$ 6.8 billion in 2019 from the report of 119 low and middle income countries that reported data (WHO, 2019).

2.1.3. Tuberculosis in Ethiopia

It is apparent that ill health in a fast growing population is an obstacle to social and economic development. To minimize this, Ethiopia has chosen to strengthen primary health care as a strategic approach to address the major gaps in public health. Although TB prevention and control programs have got more attention after implementation of DOTS strategy since 1990's, the disease still remains a major challenge of public health throughout the country. According to WHO Global TB Report 2019, Ethiopia continued as one of the 30 top high TB burden countries with best estimated incidence rate of 165 cases per 100,000 populations. To be specific, the country was ranked as the twelfth and fourth top estimated incidence rate globally and from African continent, respectively. The Ethiopian Federal Ministry of Health's hospital data also revealed TB as the leading cause of morbidity, the third cause of hospital admission, and the second cause of mortality with an estimated annual mortality rate of 32/100,000 in the country (WHO, 2014).

The Health Extension Program (HEP) was initiated in 2003 to resolve the ongoing challenges of TB. To achieve the objective, the Ethiopian government deploys two Health Extension Workers (HEWs) per Kebele who can speak the language of local people (WHO, 2009). These workers receive a training of one year that prepares them to manage operations of health posts; conduct home visits and outreach services in the community. The HEWs promote preventive health care actions; refer the cases to health centers; and identify, train, and collaborate with voluntary community health workers (CHWs). It revealed an improvement of health care worker to civilian ratios, sanitation and vaccination rates like Bacillus Calmette-Guérin vaccines (BCG) for TB prevention in children. Over all, the program is considered as to be a significant enhancement in the delivery of health services (Assefa *et al*., 2009).

Though pulmonary tuberculosis is the most common presentation in the country, extra-pulmonary tuberculosis is also at a greater rate to WHO's average global proportion. There are scarcities of evidences on extra-pulmonary tuberculosis (EPTB) and in some cases the EPTB patients are categorized as other cases due to similar clinical features and limited number of pathologists to detect. Such things increase the prevalence of EPTB that resulted a challenge to the survival of human wellbeing. Acording to WHOs 2018 report, from a total of an estimated 172,000 new TB cases notified in 2017 from Ethiopia, greater or equal to 30% were EPTB which was too high as compared to the global 14% incidence rate for EPTB (WHO, 2018).

2.1.4. Molecular epidemiology and transmission dynamics

Molecular epidemiology (ME) is a blend of molecular biology and epidemiology used to study the spread of tubercle bacilli in mini epidemics or outbreaks. It is used to analyze transmission dynamics of TB and determines risk factors for the transmission in the community. Molecular epidemiology has a great role for identification of exogenous re-infection and endogenous reactivation. Moreover, it can also be used to identify cross contaminations in the laboratory. Before the emergency of molecular methods like DNA finger printing in the mid 1980's discrimination between clinical isolates of *M. tb* was difficult. Furthermore, the spread of TB was also not precise relied on observational data or anecdotal correlations. In fact, the already existing previous screening methods of the bacterium like colony morphology, comparative growth rates, susceptibility to selected antibiotics, and phage typing could play great roles in diagnosis of the disease (Mathema *et al*., 2006).

Availability of molecular tools does not guarantee the best diagnosis for TB. It needs good knowledge and experience of using the technologies. The key aspects to select adequate molecular approach for studying TB epidemiology are the observed rate of polymorphism (stability of biomarker) and the genetic diversity of strains in the population. Thus, molecular epidemiologists have gone beyond traditional outbreak analyses and have carried out systematic studies designed to address specific epidemiologic questions or test hypotheses. Researchers might apply IS*6110* typing to quantify the relative contributions of recent and remote infection to the burden of TB disease in communities, to identify risk factors for the spread of disease, and to establish the relative frequency of re-infection (Murray and Alland, 2002).

The resurgence of TB throughout the world in relation to the emergence of drug resistance ones renewed interests in understanding TB epidemiology and pathogenesis. Interestingly, one important advance in the field of TB research is the development of these molecular techniques that assist the identification and tracking of individual strains of the bacteria. This new discipline (the molecular epidemiology) revolutionized the fields of research, prevention and control of TB by providing information on transmission dynamics. It allows differentiation between strains, assessment of the overall strain diversity of *M. tuberculosis* complex including difference by region and population, and measuring the prevalence of endemic strains (van Soolingen, 2001).

The current rates of active transmission dynamics in TB could be identified by differentiating the disease as due to recent or previous infection. Determination of whether recurrent TB is attributable to exogenous re-infection or endogenous reactivation; whether all *M. tuberculosis* strains exert similar epidemiologic characteristics in populations or not; and an understanding of transmission dynamics on a population- or group-specific level, as well as in identifying extensive transmission or outbreaks from what appear to be sporadic, epidemiologically unrelated cases (Mathema *et al*, 2006).

Strain typing of the bacteria contributes significant role to have a good knowledge of TB epidemiology. This could help to improve disease control program by providing information on transmission dynamics (Tostmann *et al*., 2008) of the bacteria and in determining endogenous reactivation versus exogenous re-infection (Dobler *et al*., 2008).

Active TB might be developed as either due to endogenous reactivation or exogenous re-infection (recent infection). To identify the disease as endogenous reactivation or exogenous re-infection one can use varieties of molecular techniques. Of these techniques, DNA fingerprinting techniques is the most commonly used one; and if the isolates from two TB episodes have the same genotype then the episode is considered as an exogenous infection; otherwise, it is defined as endogenous relapse. The approach is based on the premises that TB cases share a DNA fingerprint are epidemiologically related while cases in which fingerprints are unique due to remote infection (Lambert *et al*., 2003).

Of the few molecular studies in epidemiology, transmission dynamics and drug resistant *M. tb*, those of Northwest Ethiopia revealed new findings of phylogenetic lineages. In addition to the previously described lineage (Dehli/CAS (Central-Asian clade), H (Haarlem clade), Ural, LAM (Latin-American-Mediterranean clade), TUR, X-type, S-type, Beijing and Uganda II) the study discovered new lineages named as Ethiopia_3, Ethiopia_1, Ethiopia_H37Rv like and Ethiopia_2. It also showed a high rate of recent transmission in the study area (Tessema *et al*., 2013).

In Ethiopia, where TB infection is highly endemic, the relative importance of reactivation and recent infection of the disease is not known. Molecular epidemiology of the disease in the country is not well established which might be due to lack of laboratory facilities and their expensive cost to use and detect the bacteria at molecular level. Of course, with these limitations few molecular epidemiological studies were done. Most of the strains were investigated using IS*6110* RFLP and spoligotyping which allows neither for high resolution phylogenetic strain classification nor for analysis of transmission dynamics (Agonafir *et al*., 2010).

The study made in Northwest Ethiopia used MIRU-VNTR molecular tools which gives high resolution as compared to those of region of differentiation and spoligotyping. The tool was applied and the data confirmed a highly diverse population structure comprising thirteen phylogenetic lineages, four of which were not described so far elsewhere in the world. Furthermore, the study also indicated higher rate of recent transmission with more drug resistance strains. *M. tuberculosis* Dehli/CAS was the predominant phylogenetic lineage in the area accounting for 39% of investigated strains (Tessema *et al*., 2013). Similarly, a previously published study from the capital city of Ethiopia showed 43.5% of the same strains (Agonafir *et al*., 2010).

Of the previously undefined lineages, Ethiopia_3, Ethiopia_1, Ethiopia_H37RV-like and Ethiopia_2 were clearly shown by minimum spanning tree-based analysis. The advancement for such new investigation as compared to other previous study in the country was due to the use of valid analysis of the population structure and standardized comparisons based on advanced MIRU-VNTR that have high resolution rather than IS*6110* DNA fingerprint and/or spoligotyping analysis. Such studies imply that the actual picture of *M. tuberculosis* population diversity is largely incomplete and needs a systematic investigation in other areas of the country using the best genotyping methods (Tessema *et al*., 2013).

Dynamics of TB transmission could be determined using different forms of DNA fingerprinting. Those mycobacterial isolates with identical fingerprints are considered to be clusters and attributed to recent infection while isolates with unique fingerprints are most commonly associated with endogenous reactivation (van Soolingen, 2001). Based on these standards those few studies in the country revealed that most of the *M. tuberculosis* isolates appeared in clusters implying recent infection than endogenous reactivation.

A proportion of 45.1% investigated in Northwestern Ethiopia (Tessema *et al*., 2013) and 41.2% from Addis Ababa were found in clusters. Mihret *et al.* (2012) typed 192 isolates 88% of which

were in clustered form. Similar studies indicated comparable results regarding prevalence of clustering found in population-based studies from other countries such as Botswana (42%), South Africa (45%) and Estonia (49%) (Bruchfeld *et al*., 2002). If an individual with active TB is left untreated on time, he or she will transmit on average to about 10 to 15 people every year which could be responsible for the increment of exogenous re-infection (Dye *et al*., 2005).

2.1.5. Genotypic methods

The conventional techniques commonly used for TB diagnosis like acid-fast bacilli smear microscopy lack sensitivity; and the gold standard culture test takes time for identification of *Mycobacterium*. In addition, the diagnostic methods do not show the species level of the *Mycobacterium* (Warren *et al*., 2006). To overcome such limitations in the diagnosis of tuberculosis and provide appropriate treatments as well as to trace source of infection and arrest further transmission on time genotypic methods have been developed. It is expected that the typing methods should be rapid which do not need growth of the organism, reproducible, easy to perform, reduce biohazard risks, inexpensive and directly applicable to clinical material. In fact, because of their expensive cost and other complications, using the best typing method is rare particularly in TB endemic developing countries. Those harmonized and reliable typing methods permit easy identification of locally or internationally circulating clones, which is essential for optimal epidemiological surveillance and disease control (Palomino, 2005).

Molecular typing methods include the “gold standard” analysis of highly conserved DNA fingerprinting patterns obtained by RFLP analysis of the insertion sequence IS*6110*(IS*6110*-RFLP fingerprinting), the study of variations within the genomic direct repeat region by spoligotyping and the typing of mycobacterial interspersed repetitive-unit-variable number of DNA tandem repeats (MIRU-VNTR). These are powerful molecular epidemiological typing methods depending on the analysis of mobile DNA elements (IS*6110*-RFLP fingerprinting) or repetitive DNA elements (spoligotyping and MIRU-VNTR typing) that change quite rapidly providing a high degree of discriminatory power (Mathema *et al*., 2006). Strains of *M. tuberculosis* differ in the number of IS*6110* copies and their distribution is highly variable in the *Mycobacterium* genome. In addition,PGRS, SNP and GeneXpert are also among the typical genotypic methods which contribute considerable role in the diagnosis and determination of transmission dynamics. These molecular methods are used to identify rarely encountered *M. tuberculosis* complex bacteria (*Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium canettii*)which were previously difficult to distinguish using the existing original biochemical testing procedures (Bouakaze *et al*., 2010).

a) Region of difference (RD) deletion typing

The challenge of rapid and simple commercial assays for species identification of MTBC members is partly due to the observation that members of the complexes are 99.9% similar at the nucleotide level. In addition, they have also identical 16S rRNA sequences, the most commonly used molecular marker in microbiology for species identification. This similarity suggests that members of the bacteria are evolved from a common ancestor through sequential DNA deletions with precise genomic locations. Of the MTBC, *M. canettii* was suggestedas a potential ancestral species(Brosch *et al*., 2002; Palomino *et al*., 2007).

The complete DNA sequence of *M. tb* H37Rv has provided information on regions of difference (RD 1 to RD 16) deleted in members of the MTBC other than *M. tuberculosis* (Parsons *et al*., 2002). These regions of difference represents the loss of genetic material that arise due to errors in DNA replication, movement of mobile genetic elements, mycobacteriophage-mediated transduction, or recombination between adjacent homologous DNA fragments with loss of the intervening sequence. The *M. tb* species posses RD9 locus which is missed in most other species of the complexes. This showed that some RD loci are restricted to one species of MTBC or its subspecies, while others appeared to be differentially distributed among the complex groupings (Pinsky and Banaei, 2008).

The regions of differentiation harbor several important genes and virulence factors, and their presence or absence could help to identify species of different isolates in a particular geographical region on an evolutionary time scale. The patterns have emerged as important for typing systems in epidemiological and evolutionary studies of *M. tuberculosis*. Subsequent studies also found that some of these deletions were variable and also absent from other subspecies of the genus *Mycobacterium*. Some RDs, namely, RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, RD13 and RD14 contain the coding sequences. Deletion of these RDs may be due to rare strand- slippage errors of DNA polymerase, although the exact mechanism of such events is still elusive. Of these deletion patterns, RD9-PCR identifies *M. tb* from the other MTBC species (Brosch *et al*., 2002; Pinsky and Banaei, 2008). These deletion typing is used in cultured bacteria for differentiation of the *Mycobacterium* species. The assay is reliable with some limitations as the bacterial culture takes 2 to 8 weeks and the conventional PCR assay involves two to five separate reactions requiring post amplification processing, thereby increasing the risk of contamination (Halse *et al*., 2011).

b) Spacer oligonucleotide typing (Spoligotyping)

Spoligotyping is a widely used DNA fingerprinting methods that allows simultaneous detection and molecular typing of *M. tuberculosis* complex. It is one of the useful tools for the management of tuberculosis in clinical settings. Spoligo has a meticulous value in population based studies with low cost to define the phylogeographic specificity of the circulating clades/families of tubercle bacilli. It is recommended as the best preliminary screening test for large number of *M. tb* isolates. In fact, to assess the genetic relatedness and the epidemiological links among TB outbreak-related cases the most sophisticated and cost demanding IS*6110*-RFLP, MIRU-VNTR or whole genome sequencing typing are preferable (Gori *et al*., 2005).

Spoligotyping is based on the visualization of the spacer DNA sequences in between the 36-bp direct repeats (DRs) in the genomic DR region of *M. tuberculosis* complex strains. The DR locus was first described by Hermans *et al.*(1991) who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. This DR region contains a variable number of DRs and also a variety of spacer DNA sequences in between the DRs. On the basis of the knowledge of the DNA sequences of the spacers present in the direct repeat locus of reference *M. tuberculosis* strain H37Rv and *M. bovis* BCG vaccine strain one could easily detect and type the causative agent (Palomino, 2005; Shi *et al*., 2018).

The synthetic oligo's are designed and applied in lines on a DNA membrane. There are about 100 different spacer sequences identified in the MTBC and 43 of them have been selected for use in spoligotyping.In order to examine the presence of the 43 spacers in the DR region of an unknown *M. tuberculosis* complex strain, the whole DR locus of that strain is amplified with PCR by using two inversely orientated primers (DRa Biotin labeled and DRb) which are complementary to the sequence of the direct repeats. By using such primers, DNA in between DRs that are adjacent to each other and in between DRs are more distantly positioned and amplified. The PCR products, which are of multiple sizes, are applied on the membrane in reverse orientation on the rows with the synthetic oligo's. Since one of the DR primers is labeled (DRa) with a biotin through a streptavidine-peroxidase conjugate and a substrate, the hybridization on the synthetic oligo's can be detected by chemiluminescence (Van Soolingen, 2001).

Spoligotyping applied to culture is simple, robust and highly reproducible. Moreover, the results can be read as a digital code even in a word processor. However, the appropriateness of spoligotyping as a replacement to the gold standard IS*6110* RFLP typing is doubtful. A proportion of *M. tuberculosis* strains with marked differences in their IS*6110* RFLP patterns might exhibit identical spoligo patterns. The discriminatory power of spoligotyping to distinguish between *M. bovis* isolates is less than that of PGRS- or DR-based RFLP typing. In fact, spoligotyping can be used for pre-screening. If *M. tuberculosis* complex isolates have different spoligo patterns, they also reveal, without exception, different IS*6110* RFLPs. The ligation mediated PCR has been described to be more effective than spoligotyping for screening on large scale. This technique can be used to classify *M. tuberculosis* complex isolates in taxons or subspecies (Van Soolingen, 2001).

In general, one of the best advantages of spoligotyping over the gold standard typing is that, in principle, spoligotyping can be used simultaneously for the detection and typing of *M. tuberculosis* complex bacteria in a single assay. In addition, it has proven that spoligotyping perform typing on non-viable cultures, on slides of Ziehl Neelsen staining and on paraffin-embedded material. It also serves as a typing method for strains with less than five copies of IS*6110*. Contrary to such preferable qualities, severe problems are faced in obtaining identical spoligo patterns from bacteria in clinical material due to factors present in a proportion of clinical specimens that inhibit PCR. Although the method provides digital typing data, it is only measuring variability in a single locus and does not generally provide sufficient discrimination for outbreak investigation (Kremer *et al*., 1999).

The DR region in individual *M. tuberculosis* strains and in different members of the *M. tuberculosis* complex was identified and alignment of the spoligotype patterns was done to group the isolates according to similarity into clades or strain families. It was also applied to detect the causative bacteria and provide epidemiological information on strain identities. Furthermore, Spoligotyping was used for genotyping of the *Mycobacterium* isolates and identify the circulating lineages with cluster formation in the specified study area (Kamerbeek *et al*., 1997).

c) The mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing

The MIRU-VNTR typing is a technique with most acceptance in comparison to spoligotyping sensitivity and RFLP-IS*6110* specificity. It shows an adequate balance between variability and have also essential feature to differentiate non related isolates. The MIRU has also greater discriminatory performance as compared to spoligotyping and better performance was identified when both are combined. In fact, like spoligotyping it is useful to differentiate *Mycobacterium* strains with few copies of IS*6110* unlike that of RFLP (Jonsson *et al*., 2014; Shi *et al*., 2018)

The MIRU-VNTR typing method is essential for epidemiological purposes in investigating the spread of specific genotypes and trace TB transmission at the strain level. It is also important for determination of recent transmission among the community (Rozo-Anaya and Ribon, 2010). Variability in the numbers of tandem repeats that are 40–100 bp elements dispersed in intergenic regions of the *M. tuberculosis* genome are vital for its typing. A total of 41 loci have been identified in *M. tuberculosis* with 12 of them showing polymorphism. In MIRU-VNTR, the different strains vary in the number of allelic repeats at different loci.Presently, it is accepted that the MIRU based typing eventually replaces the classical IS*6110* RFLP typing and becomes as the major standard method for the fast and high-resolution genotyping of *Mycobacterium tuberculosis* complex isolates (Kanduma *et al*., 2003; Jonsson *et al*., 2014).

The 24-locus sets of MIRU-VNTR have improved discriminatory importance than the initial 12-locus or the 15-locus sets and is suggested as the current gold standard technique in the molecular typing of *Mycobacterium tuberculosis* complex isolates (Supply *et al*., 2006; Iwamoto *et al*., 2007; Beer *et al*., 2014). In population-based studies, standard MIRU-VNTR typing was shown to have an equal and rather slightly better predictive value than the prior gold standard IS*6110* RFLP which is time consuming, technically demanding and labor intensive . The MIRU technology is crucial for the study of TB transmission, in settings with epidemiological characteristics representative of developed countries (Supply *et al*., 2006). The method primarily involves PCR amplification followed by the detection and sizing of PCR fragments using either capillary electrophoresis on automated sequencers or using the agarose gels (Nikolayevskyy *et al*., 2016).

Implementation of the method requires the ability to accurately size the resulting amplicons. Analysis by traditional gel electrophoresis is tedious and slow and MIRU typing has so far been largely restricted to laboratories with sophisticated high throughput analytical facilities such as an automated sequencer (Supply *et al*., 2001). The capital outlay, maintenance costs and training requirements are also beyond the budget of many public health laboratories, particularly in those countries where TB is endemic. Thus, the cost-effective gel electrophoresis method would enable greater implementation of MIRU-VNTR to aid the control of TB.

2.2. Drug Sensitivity Testing of *Mycobacterium tuberculosis*

Antimicrobial susceptibility testing is vital in prescribing an effective drug regime for a tuberculosis patient, especially in areas where drug resistance incidence is high like Ethiopia. Emergence of the drug-resistant bacteria (multidrug resistant and extensively drug resistant) is usually a man-made problem, largely being the consequence of human error as a result of individuals or combination of factors related to management of the drug supply, patient management, prescription of chemotherapy, and patient adherence (FMOH, 2009; Biadglegne *et al*., 2014). Globally, incomplete treatment of the cases caused over 200,000 individuals as drug resistant in 2015 (WHO, 2017). Naturally occurring genomic mutants of the bacteria also cause resistance and transmitted within the people. Such conditions lead to about 600,000 new cases and 240,000 deaths every year due to MDR or RR. The extensively drug resistant TB has also been its own share and reported by 123 countries globally with about 6.2% of patients (WHO/CDS/TB/2018.5; Zhang and Yew, 2009).

It was also during the first half of 20^{th} century that the problem of TB emerged as an insoluble challenge which was believed as due to the lipid-rich cell envelope of the bacteria. This depressing view of the *Mycobacterium* was confirmed when the first antibiotics developed (sulfonamides and penicillin) had no useful activity against it. The disease control and treatment was increasingly challenging after dramatic outbreaks of MDR-TB in the early 1990s (Nachega and Chaisson, 2003). In fact, mid of 20^{th} century was the bright time that tuberculosis chemotherapy emerged as successful for the first time (Daniel, 2006).

In recent years after a gap of more than half a century without any new TB drugs, the U.S. Food and Drug Administration (FDA) approved bedaquiline as a new drug in 2012 for the treatment of the challenging MDR-TB. Using this drug reduces treatment periods of MDR-TB from 24 months to six or nine months (WHO, 2017). Additionally, Delamanid was approved as another new life saving drug by EMA for those encountered by drug resistant bacteria. Unfortunately, strains resistant to this new molecule have also been reported (Segala *et al*., 2012; Wallis *et al*., 2016).

The detection of TB case on the spot of diagnosis is fundamental to minimize its transmission rate and the burden of treatment particularly to those of drug resistant ones (Aktas *et al*., 2014; WHO, 2015). However, wrong diagnosis and treatment leads to complications of morbidity, mortality, and further transmission of the infection. For such diagnostic challenges WHO recommended a rapid molecular Xpert MTB/RIF test in 2010 that plays greater task for initial diagnostic test in people at risk of drug resistant ones. The use of LPA was also an additionally valuable tool to detect MDR-TB in comparison to the time taking existing conventional culture methods (Banu *et al*., 2014). Later on, the "End TB Strategy" also calls as early diagnosis and prompt treatment for all persons of all ages with any form of drug susceptible or drug resistant TB. This requires ensuring access to WHO-recommended rapid diagnostics and universal access to DST for all patients (not only for those at risk of drug resistance or HIV associated TB cases) with signs and symptoms of TB. WHO further defines a universal access to those drug sensitivity testing technologies of MGIT and LPAs (WHO, 2014).

2.2.1. Socioeconomic impact of the drug resistant *Mycobacterium*

Though the mortality rate of TB is reducing by 3% annually, the threat of its drug resistance is increasing (WHO, 2017). The cumulative effects of treatment interruption like lack of awareness about the nature of bacteria, shortage and lack of WHOs recommended diagnostic tools, and prolonged drug consumption period for treatment increases the risk. Unless the new drug regimens (Bedaquiline and Delamanid) are used, MDR-TB treatment could take as long as two years and even more causing longer absenteeism from work or even loss of employment, social isolation, and long-term socioeconomic and psychological effects. Routinely performed activities like cooking, cleaning, childcare, and managing most activities of the household are also affected when women are attacked by the disease. Additionally, lose of income generating economically active segment of the population due to TB also scale up the risk and things become more complicated for the cases family members (Morris *et al*., 2013).

Although TB drugs are cost free for most high TB burden countries including Ethiopia, the patients face other health related charges for treatment. The long-suffering drug resistant TB cases face extra charges as 5-20 times than the susceptible ones. This is due to relocation costs, longer pre-diagnosis and treatment periods, involvement of more health institute visits with further procedures for its diagnosis. Both direct costs (admission fees, paying for laboratory smear test and X-ray, costs for better feedings, transportation to and from the health institute) and indirect costs like loss of income due to sickness causes greater socio-economic burden particularly among drug resistant cases (van den Hof *et al*., 2016). WHO surveys conducted in Myanmar and Viet Nam approved the reality of these high economic and financial burdens due to TB (WHO, 2012).

2.2.2. Global incidence, prevalence and mortality due to *M. tuberculosis*

In 2018, an estimated 3.4% of the global TB cases were new drug resistant (MDR/RR) and 18% were among previously treated cases. The greatest estimation of new MDR/RR TB cases were observed in Europe (18%) and less in Americas and African continent with a proportion of 2.5% each. In Ethiopia, the estimated incidence of MDR/RR is 0.71% and 16% among new and previously treated cases, respectively. This percentage is in less proportion than the global incident rates of the year (WHO, 2019). Although diagnosis and successful treatment of people with TB are ever more increasing for the last 10 consecutive years, there are still large and persistent gaps in detection and treatment of the cases. There is also no as such visible changes of the new and previously treated drug resistant cases annually (Table 1).

Table 1. Global trends of TB incidence, drug resistance and TB related deaths for the last ten consecutive years (2009 - 2018)

Year	**Estimated TB incidence in million**	**Estimated % of MDR/RR-TB cases**		**TB related death in million**	**Reference**
		New	Previously treated		
2009	9.4	3.3	21.0	1.7	WHO, 2010
2010	8.8	3.4	20.0	1.5	WHO, 2011
2011	8.7	3.7	20.0	1.4	WHO, 2012
2012	8.6	3.6	20.2	1.3	WHO, 2013
2013	9.0	3.5	20.5	1.5	WHO, 2014
2014	9.6	3.2	18.0	1.5	WHO, 2015
2015	10.4	3.9	21.0	1.8	WHO, 2016
2016	10.4	4.1	19.0	1.7	WHO, 2017
2017	10.1	3.6	17.0	1.6	WHO, 2018
2018	10.0	3.4	18.0	1.5	WHO, 2019

In 2018, the estimated cases of drug resistant TB (MDR-RR) had a magnitude of 484,000 which was about 10% downward from the best estimate published by WHO in its 2018 global TB report. Of these estimated cases, about 44.2% (214,000) deaths were due to MDR/RR-TB which was also a downward revision to the best estimates. In fact, the global notified cases rather than estimates of MDR/RR-TB were 186,772 up from 160,772 cases in 2017, and 156,071 cases were enrolled in treatment which was also up from 139,114 in 2017. The number of people enrolled in treatment in the year was equivalent to only 32% of the estimated incidence of the 484,000 cases. The overall global reduction in the total number of TB deaths between 2015 and 2018 was 11% which is less than one third of the way towards the End TB Strategy milestone of a 35% reduction by 2020. Thus, further measures should be considered to improve the coverage and quality of diagnosis, timely treatment and care for people with drug-resistant TB to meet the estimated end TB targets of the 2035 (WHO, 2018; WHO, 2019).

In Ethiopia, the first MDR-TB patient was admitted to St. Peter's Hospital by 2009 in a rehabilitated isolation ward. Since then its prevalence was progressively increasing at an alarming rate and the country became as one of the top drug resistant countries globally within a short period of time (FMOH, 2009). Now days, best improvements are registered in all aspects of the three parameters (incidence, prevalence and mortality rate) attaining the WHO's 2015 "end TB goal" and promising to the 2035 global "End TB targets". TB mortality rate is also falling down by 12% per year in Ethiopia as compared to the global 3% mortality rate (WHO, 2017).

The challenge of drug resistant TB in different parts of the country and data on patterns of resistance among Ethiopian isolates vary. Of course, with all these progress in comparison to most sub-Saharan African countries the prevalence of MDR bacteria is still high. Surveys conducted in Ethiopia by 2005 found that the prevalence of MDR-TB to first line drugs (isoniazid) and (rifampicin) among exogenous re-infection and endogenous reactivation was 1.6% and 12.0%, respectively (WHO, 2010). Similarly, there was also a comparable drug resistance TB among retreatment cases than the new ones after ten years with an estimate of 0.71% in new and 16% in previously treated TB cases of the 2018. This revealed that drug resistance is more common in previously treated TB cases than the newly infected ones without any visible reduction of drug resistant TB realizing further threats of the disease in the country (WHO, 2019).

2.2.3. Treatment and *M. tuberculosis* drug resistance

Treatment

Tuberculosis, the leading cause of mortality throughout the world, is a curable disease if properly treated and right measures are taken on the spot. The golden rule in prevention and treatment of the disease is understanding nature of the bacteria and its means of transmission. Enhancing community awareness, rapid accurate diagnoses under quality laboratories, immunization with

TB vaccine at childhood and correct use of drugs on time play major roles in the prevention and control programs. Mobilizing the societies for immunization program, proper opening of rooms to get fresh air and allowing direct light, use of masks and respirators could minimize the risk of transmission. Furthermore, follow up of patients for completing their treatments and tracing defaulters could minimize the risk of drug resistant bacteria. Those who have close contact with the patients should also be checked for active bacteria and the patients must be screened and isolated in high risk environments to prevent the progress of transmission. Appropriate polices are needed for effective clinical and public health management with committed and coordinated efforts from within and outside the health sector (WHO, 2012).

Against intensive research and measurements going on to solve the overall impact of TB, there is still an increase of MDR and XDR bacteria which causes major threat to effective TB control. The World Health Assembly (WHA) in 1991 considered particular attention to the disease burden when TB was recognized as a major public health problem. WHO also declared TB as a "global health emergency" in 1993 with the DOTs program development in 1994 to combat TB and minimize the disease complication. Nevertheless, complications of TB with HIV and emergence of drug resistant TB inhibited the targeted achievement of the planned global STOP TB program (Klopper *et al*., 2013).

The disease treatment involves the administration of a number of drugs for longer duration. The most popularly used treatment dosage regimen contains 2HREZ/4HR3 (Isoniazid, Rifampicin, Ethambutol and Pyrazinamide daily for two months, followed by four months of Isoniazid and Rifampicin given three times a week) in two phases. During the initial intensive phase of treatment, which lasts for 2 months, patients are treated with combination of drugs to minimize the emergency of drug resistance. In the following four months of the continuation phase, the most potent anti-tuberculosis drugs (INH and RIF) are given to kill any of the persisting *Mycobacterium* organisms. Tuberculosis treatment becomes difficulat due to resistance to all the four primary drugs-INH, STM, EMB and RIF-(Aktas *et al*., 2014; Somasundaram *et al*., 2014).

In relapsed TB cases an eight month regimen containing STM-RMP-INH and EMB is prescribed for the first two months followed by INH-RMP-PZA-EMB for one month during the intensive phase. Through the next five months of the continuous phase INH-RMP-EMB are recommended to be used (WHO, 2010).

Recently, for non-complicated MDR tuberculosis, WHO endorsed a treatment regimen of 9 to 12 months as an alternative to the two years conventional regimens. Fluoroquinolones (Levofloxacin (CC), Moxifloxacin (CC), Moxifloxacin (CB) and Gatifloxacin (CC)), second-line injectable drugs (kanamycin, amikacin and capreomycin), clofazimine and bedaquiline, cycloserine and terizidone, linezolid and the delamanids are the key components for these shortened regimens (WHO, 2018). The injectable drugs for drug resistant *Mycobacterium* could be stopped when sputum cultures are negative (Calver *et al*., 2010). The development of new drug pipelines is progressing but not as fast as the bacterial challenges. Recently, as part of efforts to improve outcomes for MDR/XDR-TB, 90 countries and territories reported that they had started using the new drug bedaquiline and 57 reported they had started using delamanid by the end of 2018 for tuberculosis treatment (WHO, 2019). Beside all the cases treatment with the existing drugs, some of the *Mycobacterium* are still mutating and come into view as drug resistant.

Drug resistance

In 1990s, multidrug resistant ***Mycobacterium*** (resistant to at least INH and RIF) was emerged for the first time after identification of single drug resistance. Soon after, extensively drug resistances (XDR) TB come into view in 2001 which is resistant to fluoroquinolone and one of the three second line injectable drugs. Recently, the so called total drug resistance (TDR) *Mycobacterium* was further identified as a severe untreatable TB causing bacterium in 2007 (Banerjee *et al*., 2008; Haydel, 2010; Slomski, 2013; Velayati *et al*., 2013). Emergence of these adverse drug-resistant TBs bring severe socio-economic burden mainly in developing countries and convey extraordinary task to TB treatment and control programs. Treating drug resistance forms of TB are more difficult in immuno-compromised patients and pose serious threats to the global health.

The extent of resistance associated with the Beijing GenoType makes treatment options extremely difficult. The isolates are resistant to all first-line anti-TB drugs and also to many of the second-line drugs. This suggests that the Beijing GenoType clone is evolving toward total drug resistance. The presence of widespread drug resistant bacteria is making TB prevention and control as more complicated (Velayati *et al*., 2009; Adetutu and Nafiu, 2013).

In Ethiopia, in order to facilitate the management of TB all of the five drugs (STM, INH, RIF, PZN and EMB) are used as anti TB. These drugs are available in loose and fixed dose combination (FDC). The FDC drugs exist in the form of 2FDC and 3FDC formulations. Ethambutol and isoniazid, rifampicin and isoniazid were categorized as the 2FDC, where as rifampicin, isoniazid and pyrazinamide are available as 3FDC. Three different categories of treatment regimens are available in the country and each regimen is recommended for a defined group of patients and their formulation includes the duration of treatment, type of drug and frequency of delivery. Drug susceptibility profile is unstable in the country due to the frequent gain of drug resistance. Each of the TB drugs has target structures in the *Mycobacterium.*

Anti-TB drugs, mechanism of action and resistance development

Rifampicin is the drug that had long been believed to target the *Mycobacterium* RNA polymerase and thereby kill the organism by interfering the transcription process or inhibiting RNA synthesis (inhibits transcription of DNA to RNA and subsequent translation to proteins). This drug should always be used as an anti-tuberculosis treatment, unless the patient has an isolate resistant to it. It is regarded as a surrogate marker for MDR-TB, since greater than 90% of isolates resistant to rifampicin are also resistant to isoniazid. RIF resistance is caused by point mutations or nucleotide deletions or insertions in an 81-base pair core region (codon 507 to 533) of the *rpoB* gene, which codes for β-subunit of DNA-dependent RNA polymerase. over 95% of all RIF resistant isolates have a single mutation in this 81-base pair region of the *rpo*B gene and greater than 92% of the mutations occur at either codon 516 (codes low level resistance), 526, or 531 (which codes for high level resistance) (Arnold *et al*., 2005; Sandgren *et al*., 2009; Somasundaram *et al*., 2014).

Isoniazid is an important form of drug to form MDR with rifampicin. It blocks the biosynthesis of cell wall mycolic acids in *M. tuberculosis*, thereby making the *Mycobacterium* as susceptible to reactive oxygen radicals and other environmental factors. This component of the cell wall also prevents the bacterium from chemical damage, dehydration and effective activity of hydrophobic antibiotics. In addition, mycolic acid allows the bacterium to grow readily inside macrophages, effectively hiding it from the host’s immune system. Once circulated in the bloodstream and enters the cell by passive diffusion, the isoniazid prodrug is activated via a bacterial catalase-peroxidase enzyme, encoded by *katG* gene (Whitney *et al*., 2002). The activated INH principally targeted to the NADH specific enoyl-acyl carrier protein reductase (*inhA*) and a beta ketoacyl-acyl carrier protein synthase which is involved in mycolic acid synthesis necessary for the mycobacterial cell wall. Depletion of this acid leads to the bacterial killing. Unlike that of RIF resistance which concentrated on one gene (*rpoB)*, additional mutation of other genes (*acp*M, *ahp*C and *kas*A) exist for INH but with minor role. While *katG* mutations (at codon 315) may confer high-level isoniazid resistance, *inhA* mutation may cause low-level of isoniazid resistance and also cross resistance to ethionamide (Timmins and Deretie, 2006; Silva and Palomino, 2011).

Pyrazinamide: like INH it is also a prodrug that stops the growth of *M. tuberculosis*. PZA kills a non-replicating dormant *M. tb* under acidic conditions that other TB drugs fail to do so, and thus making it an essential drug. It diffuses into the granuloma of *M. tuberculosis*, where the tuberculosis enzyme pyrazinamidase converts pyrazinamide to the active form pyrazinoic acid which is bactericidal. Resistance to this drug is mostly caused by mutations in the *pnc*A gene encoding pyrazinamidase involved in conversion of the prodrug PZA to the active form. Mutations in the drug target *rps*A are also found in some PZA-resistant strains. Findings also showed that *pan*D gene mutations are found in some PZA-resistant strains without mutations in *pnc*A or *rps*A genes. This suggests that a third PZA resistance gene and a potential new target of PZA (Zhang *et al*., 2013). Naturally, in contrast to the other members of *M. tuberculosis* complex, *M. bovis* is resistant to pyrazinamide (a drug used for shorter course of treatment) and it could serve as an intial screening tool for *M. bovis* (Valle *et al*., 2015).

Streptomycin: a broad spectrum drug that interferes with prokaryotic protein synthesis through binding to ribosomal protein. Although it has been used as an important drug since its discovery, increased frequency of its resistance by *M. tb* limits its therapeutic efficacy. STM can be used interchangeably with EMB when the bacteria are susceptible to it. Mutation to *rps*L locus of the bacteria causes resistance. High level resistance mainly involves mutation in *rps*L codon 43 (*rps*L43) and *rps*L 88 while mutation in the *rrs* gene cause low level resistance (Abbadi *et al.*, 2001).

Ethambutol: a TB drug administered orally and prevents cell wall synthesis through inactivation of the enzyme arbinosyl transferase causing impairment of cell metabolism and finally leading to cell death. The probability of *emb*B gene mutation is directly proportional to the mycobacterial load (Arega, 2007; Goude *et al.*, 2009).

With all the indicated commonly used first line drugs the threat of TB is increasing due to the bacterial drug resistance nature. Thus, the two new drugs bedaquiline and delamanid and growing evidence for the use of linezolid, offer renewed hope for addressing MDR-TB. However, *M. tb* also develops resistance to this new generation drugs (bedaquiline and delamanid). Bedaquiline (BDQ) inhibits the normal function of the enzyme ATP synthase inside *M. tuberculosis* and the specific loss of this enzyme activity kills the bacteria. However, its resistance was developed as the genome is sequenced and showed mutation in *mmp*R gene. Bedaquiline resistant strain also up-regulate *mmp*L5 (multisubstrate efflux pump), *atp*E gene mutations and *pep*Q mutation. Likewise, delamanid (DLM) also developed mutations in *fbi*A and *fgd*1 (Andries *et al*., 2014; WHO, 2017). Delamanid blocks the manufacture of mycolic acid.

2.2.4. Phenotypic and genotypic drug sensitivity tests

The most commonly used TB testing method (microscopic smear test), chest radiography and clinical presentations cannot detect drug sensitivity. Instead, a time taking process (4-8 weeks) conventional culture method and the lately WHO recommended rapid GeneXpert diagnostic test are primarily used for sensitivity test (Outhred *et al*., 2015). The phenotypic bacterial growth indicators using liquid cultures (MGIT) and the genotypic bacterial DNA detection using LPA can also be used in the detection of drug sensitivity (Maningi *et al*., 2017).

A) Phenotypic drug sensitivity tests

Phenotypic DSTs are the methods based on cultivation of *M. tb* from clinical samples in the presence of antibiotics either on solid or liquid medium. The solid culture media (LJ) is cheaper and more commonly applied technique for over 100 years. However, it is labor intensive, less sensitive and time taking than the liquid culture (BACTEC MGIT 960). The liquid bacterial cultivation method is an automated technique that process large numbers of specimens at a time but with more prone to contamination. Thus, the shifting of solid culture to liquid ones needs more curiosity (Lawson *et al*., 2013). Both methods are validated against clinical outcome at least for the first line drugs and also established for most second line drugs, although the breakpoints to predict clinical susceptibility for some of those drugs are not as well characterized (Schon *et al*., 2009).

The delay of results for treatment in such conventional diagnostic method enhances the chance of disease transmission that is harsh specifically for the drug resistant ones. Drug susceptibility detection method involves the absolute concentration, resistance ratio and proportion method.

a) The absolute concentration method: In this method, the drug is incorporated into two-fold dilutions of each drug and resistance is indicated by the lowest concentration of the drug which will inhibit growth (Drobniewski *et al*., 2007).

b) The resistance ratio method: variations in the minimum inhibitory concentration (MIC) are used to determine the drug susceptibility. The resistance ratio of MIC is calculated by dividing the MIC of test isolate to the MIC of standard susceptible strain (H37Rv) and interpreted as susceptible, low level or intermediate DR and high DR when the resistance ratio level is less or equal to 2, 4, 8 or more, respectively (Drobniewski *et al*., 2007).

c) The method of proportion (MOP): is a universally accepted gold standard method used to detect drug sensitivity but requiring 4 to 6 weeks to get a final result that increases the risk resistant transmission (Aktas *et al*., 2014; Banu *et al*., 2014; Aung *et al*., 2015). MOP is calculated by counting the number of colonies grown on drug containing medium to colonies grown on drug free medium at a defined critical concentration. In this case, resistance is defined when the proportion is greater than 1% (Kalokhe *et al*., 2013).

d) BACTEC MGIT 960: is rapid phenotypic drug sensitivity testing method that uses an oxygen-quenching fluorescent sensor technology in conjunction with unique algorithms to determine positivity of the culture tubes. Its medium is a tube containing Middlebrook 7H9 Broth that supports the growth and detection of mycobacteria (Telles *et al*., 2002). The tube also contains a fluorescent compound embedded in silicone at the bottom of a 16 x 100 mm round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, the actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. The emission is recorded by an automated instrument BACTEC MGIT 960 System. This method is used to determine susceptibility to first line drugs STM, INH, RIF and EMB (Scarparo *et al*., 2004).

B) Genotypic drug sensitivity tests

The conventional procedures for the isolation of *M. tb* and DST are slow, causing substantial delays until patients with drug-resistant TB receive adequate treatment. To overcome this and further transmission of the resistant ones, molecular based anti-tuberculosis drug resistant bacteria have recently been elucidated. This detection method is based on targeted gene mutation of the bacteria against the antibiotics. But the technique detects both live and dead bacteria so that positive result does not imply the viability of the pathogen as its draw back and thus the method cannot be used for monitoring treatment response (Friedrich *et al*, 2013).

a) GeneXpert®MTB/RIF TB assay (Cepheid, CA): It is a molecular beacon-based RT-PCR assay with high sensitivity (86–100%) and specificity (95–100%) for the detection of RIF resistance in clinical specimens (Boehme *et al*., 2010). Sample processing using the instrument involve bacterial lysis, nucleic acid extraction, amplification, and amplicon detection of the target sequence with result interpretation in a closed well to prevent cross contamination. The primers in the GeneXpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with resistance to RIF. When there is exact nucleotide concordance between the probe and target sequence, the beacons emit fluorescent signals. Lack of signaling suggests that there is a mutation of the targeted gene in the bacterial DNA (Kalokhe *et al*., 2013).

Early detection of RIF resistance within less than 2 hr's of the specimen collection is as an advantage by GeneXpert®MTB/RIF or the recently launched Xpert® MTB/RIF Ultra (WHO, 2017). However, the instrument is expensive and it detects only RIF resistant bacteria as its limitation. Lack of live and dead bacterial discrimination by the instrument also makes it as not appropriate for the surveillance study of drug resistance studies (Schon *et al*., 2009).

b) PCR based solid-phase reverse hybridization line-probe assays (LPAs): The utilization of LPA was endorsed by WHO in its 2008 policy statement for smear positive pulmonary specimens to detect drug sensitivity (WHO, 2008; Ando *et al.*, 2010). The assay uses multiplex PCR amplification and reverse hybridization to identify *M. tb* complex and mutations. The LPA can be performed within 1-2 days having high sensitivity and specificity for detection of RIF resistance (≥97% and ≥99%) and INH resistance (≥90% and ≥99%) on culture isolates and smear-positive sputum, respectively. The overall agreement of LPA and conventional DST for detection of MDR-TB is great with a proportion of 99% (Ling *et al*., 2008).

The three commercially available line probe assays for the detection of first-line drug resistance *M. tb* are the INNO-LipA® Rif.TB (Innogenetics, Belgium), GenoType® MTBDR, and second generation GenoType® MTBDR*plus* (Hain LifeScience GmbH, Germany). The *rpoB* (coding for the β-subunit of the RNA polymerase) gene mutation responsible for RIF resistance is detected by all the three assays. GenoType® MTBDR additionally detects *katG* mutations; and GenoType® MTBDR*plus* detects both *katG* and *inhA* mutations of INH (Brossier *et al*., 2009; Aung *et al.*, 2015). For detection of INH resistance, the *katG* gene (coding for the catalase peroxidase) and the promoter region of the *inhA* genes (coding for the NADH enoyl ACP reductase) are examined.

c) Sequencing: provides more detailed information and detects the most common mutations involved in drug resistance (Wilson, 2013). Technologies for sequencing and analyzing the whole genome of *M. tb* have become available to guide physicians on the treatment selection for patients with drug-resistant TB (Niemann and Supply, 2014). In fact, the technology is extremely expensive and also requires expertise as its limitation.

2.3. Tuberculosis and Malnutrition

Malnutrition refers to both undernutrition and overnutrition. Individuals are undernourished if their diet does not provide adequate calories and protein for maintenance as well as growth or if they cannot fully utilize the food they eat due to illness. Even if people get enough to eat, they will become undernourished if the food they eat does not supply the proper amounts of micronutrients to meet daily nutritional requirements. Undernutrition includes stunting (low height for age), wasting (low weight for height), underweight (low weight for age) and micronutrient deficiencies or insufficiencies (a lack of important vitamins and minerals) (Soeters *et al.*, 2008). Hence, assessment of nutritional status in an area is important since in one way or another it alters the immune profile of an individual and make vulnerable to various diseases.

Globally, more than 800 million people are chronically undernourished. Nutritional status of an individual has a substantial role to strength the immune system. Malnutrition is often the result of many inter-related factors and influenced by food intake, quantity and quality of food, and health status of the individual. Its spectrum ranges from obesity to severe malnutrition.Purpose of nutritional assessment is toidentify individuals or population groupswho are malnourished, who are at risk of becoming malnourished and those who are susceptibility to disease causing agents (Ivers *et al*., 2009).

M. tuberculosis and parasitic infections affect nutritional status of a person by increasing energy requirements, reducing food intake, and adversely affecting nutrient absorption and metabolism. Malnutrition with the indicated diseases has considerable magnitude in most parts of the developing regions. The relative risk of tuberculosis among persons in the lowest body mass index (BMI) category is five-fold higher than the group in the highest BMI category.There are other studies indicating that incidence of tuberculosis are unusually high among undernourished people. In fact, malnutrition affects cell-mediated immunity (CMI) which is the principal host defense against TB. The likelihood of developing primary or latent TB infection to active disease is high in undernourished individuals.WHO (2013) recommended that patients with TB should be nutritionally assessed and receive nutritional care and support. This showed that nutrition screening, assessment and management is an integral component of TB treatment and care. Hence, it is critically important to consider the status of malnutrition in tuberculosis and parasitic prevalent areas for proper treatment and management of the disease (Cegielski and McMurray, 2004;Gupta *et al*., 2009).

2.4. Mechanisms of Immune System and Parasitic Co-infections in TuberculosisPatients

The *Mycobacterium* transferred in bronchi and alveolus primarily make contact with alveolar macrophages and dendrite cells. Infection starts with a designation of structural components of Mycobacterial cellwall (Mishra*et al*., 2011). The macrophage receptors are involved in the recognition of the cell wall structures. Interaction of *M. tb* cell componentswith these receptors activate various mediators ofinherited immunity, including TLR2, TLR4, andTLR9, which are involved in phagocytosis (Bafica*et al*., 2005).

The bacterium swallowed by macrophages are transferredin vacuoles, called phagosomes, that merged withmature lysosomes. As a result, the bacteria appear in anunfavorable environment like low pH, proteolyticlysosomal enzymes, antibacterial peptides, nitricoxide, and oxygen radicals. The bacterial defense mechanisms under these conditions represent phagosome inhibition and lysosome merging, alkalization of phagolysosomes content, and the secretion ofvirulence proteins. Accordingly, while transferred in macrophage, *M. tb* starts the cross communicationprogram with a host cell; macrophages in turn cause the synthesis of various factors of inherited immunity,followed by reprogramming in the bacterial genome,including the expression of new genetic systems and these genes represent virulencefactors. Comparative transcriptome analysis of initial and reprogrammed *M. tb* genomes might allow us togive a response, including the genes involved in this process; proteomic analysis suggests a wide variety ofthese genes products. In the case of *M. tb* survival, they are released from lysosomes into the cytosol outsidethe fractured macrophages and they infect new cells.The surviving bacteria are reproduced in alveolarmacrophages and dendrite cells and induce cytokineformation representing the peptides involved in inflammatory processes (Ahmad, S., 2011).

Crucial for the defense from *M. tb* is the activation of the immune response of the Th1 type and the production of cytokines(TNF_α, IL_2, IFN_γ, IL_6, and IL_12). The TNF_α mediates the earlyinflammatory response to the pathogen presence; and stimulates IL_1 andIL_6 production. The conclusive role in the regulation of T cell response to *M. tb* infection belongs to IFN_γ, which is produced by activated T cells, called NK cells, andmacrophages. The production of a significant quantityof nitric oxide and the differentiation of immune cells was directly related to IFN_γ functioning. It was demonstrated that nitric oxide provided *M. tb* death in mononuclear phagocytes in MT infected mice (Macmicking*et al*., 1997).IL_6 is a cytokine with a wide spectrum of functions necessary at early inflammation stages; it isinvolved in the development of T and B_lymphocytereactions. Its absence cause a delay in IFN_γ production in lung anddisease progression (Saunders*et al*., 2000). In addition, IL_6 and IL_12 play the key role in the development of the anti-mycobacterial response of Tlymphocytes and IFN_γ mediated reaction against *M. tb* after vaccination.Cytokine IL_12 also provides the development of the Th1response and the rapid accumulation of dendrite cellsvia TLR interaction with *M. tb*. The activation of IL_12production depends on TLR9 dendrite cells and TLR2 macrophages. An IL_12 deficit enhances human sensitivity to *M. tb* infection (Pompei*et al*., 2007).

Besides, *Mycobacterium* uptake by macrophages is accompanied by theproduction of several chemokines. The chemokines provide for the migration of monocytes, dendrite cells, activated macrophages,neutrophils, and T_lymphocytes in bronchoalveolar space in the case of lung tuberculosis, which represents a predisposition of granuloma formation. The dendritic cells activate TLR signaling; monocytes differentiate in macrophages producing bactericide substances, including TNF_α, which are able to regulate the *Mycobacterium* reproduction (Algood*et al*., 2003).

Studies examining the ways in which some infectious diseases interact identify a troublesome synergistic relationship which enhances susceptibility to or worsens the prognosis of the other (López-Gatell *et al*., 2007). Intestinal parasitic infections and tuberculosis exhibit an extensive distribution with a substantial medical and public health concerns in Ethiopia. Limited epidemiological studies found evidence that indicate chronic parasitic infection may increase the risk of tuberculosis and also reduce the effectiveness of Bacillus Calmette–Guérin vaccine (Elias *et al*. 2006; Alemayehu *et al*., 2014).

Asymptomatic infection of helminths in active TB cases is associated with increased regulatory (Tregs) and T-helper cell type 2 (Th2) responses and sputum smear positivity. In line with this, a rise of eosinophiles and IgE levels during helminth infections also alters the host immunity against TB. On the contrary, protection against tuberculosis is associated with enhanced T-helper cell type 1 (Th1) immune responses whereas susceptibility to the disease is associated with reduced Th1 type responses. Th1 cells produce IFN-γ that express cytotoxicity, activate macrophages and promote cell mediated immunity where as Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and they inhibit macrophage functions unlike Th1(Korn *et al*., 2009; Blackwell *et al*., 2011; Brighenti*et al*., 2012; Abate *et al*., 2015).

Resende *et al.* (2007) identified concomitant intestinal helminth infection in patients with newly diagnosed TB skews their cytokine profile toward Th2 responses, which could favor persistent *M. tb* infection and a more protracted clinical course of the disease. Such influence of helminth infections on the immune profiles of TB cases was by evaluating both cellular phenotype and cytokine profiles. The result of his study showed 27% of TB patients enrolled were co-infected with at least one intestinal helminth. At baseline, absolute frequencies of leucocytes, monocytes and eosinophils from TB and TB + Helm patients differed from those of healthy subjects.

Hence, parasitic infections elicit a profound Th2 immune response and increased IgE antibody levels with subsequent suppression of Th1 Immune response which is vital against tuberculosis (Blackwell *et al*., 2011). Pedersen and Fenton (2006) showed that helminth infections reactivate even latent TB infections and disease expression. Furthermore, several other conditions like *Diabetes mellitus*, malnutrition, and malignancies are also known to increase the risk of progressing latent TB infection to active ones (Dooley *et al*., 2009; Lonnroth *et al*., 2009).

As higher prevalence of *M. tb* parasitic co-infection is identified from an area, anti-parasitic chemotherapy should have to be implemented prior to immunization which may greatly enhance the efficacy of BCG vaccination (Li and Zhou, 2013). Higher prevalence of intestinal parasites increases morbidity of TB so that continual stool analysis and treatment are needed. Both of the diseases were shown to be as risk factors for each other due to suppression and shifting of the host's immune system. These independent immune-modulation determine the pathogencity and outcome of both parasitic infections and tuberculosis infections. Thus, having knowledge of parasitic-*M.tb* co-infection status is vital to minimize the complexity of prevention and control program of both diseases (Alemayehu *et al*., 2014; Alemu and Mama, 2017).

2.5. Knowledge, Attitude and Preventive Practice

Increasing communities' awareness is considered as an important component for early diagnosis, prevention and control program of TB to achieve the targeted "End TB Strategy" (WHO, 2016). According to Dzeyie *et al*. (2019) study on KAP relating to tuberculosis, most TB patients had better knowledgeregarding tuberculosis symptoms, mode of transmission, and correct duration of treatments. Majority of the patients had also better attitude in covering their mouth while coughing or sneezing and on safe disposal of sputum but less practical activities for their correct sputum disposal. It was also found that there was moderate knowledge and practice with poor attitude toward tuberculosis in community based study in Malaysia (Salleh *et al*., 2018). An assessment done on healthcare workers revealed a better practice regarding TB (Alotaibi *et al*., 2019).

In Ethiopia, community-based study on KAP in eastern part of the country showed the communities have basic awareness toward TB. Majority of the TB patients visiting public health facilities received information about the disease but their practical knowledge, attitude and practice was not as such comparative with the information that they had received (Tolossa *et al*., 2014; Kasa *et al*., 2019). A study made on Ethiopian prisoners in northern part of the country also showed only about a quarter of the prisoners knew the basic elements regarding TB, 41% of them had favorable attitudes, and 55% had a good practice of tuberculosis (Adane *et al*., 2017).

3. Rationale of the Study

TB is one of the important diseases in Ethiopia. Although there is a shortage of information on the molecular epidemiology of TB in the Ethiopia, a few studies which were carried out in some parts of the country revealed the presence of novel strains of *M. tuberculosis* in the country. Thus, additional studies are required in order to establish the molecular epidemiological picture of the disease in Ethiopia focusing on regions and zones where no study has been conducted so far. The present study was conducted in the northeastern part of Ethiopia in the Amahara Regional State as no study had been conducted on the molecular epidemiology of TB before this study.

Parallel to the molecular epidemiological studies, investigation of the drug sensitivity profiles of *M. tuberculosis* plays great role in the control program of the country. It is known that Ethiopia is one of the countries with high burden of drug resistant tuberculosis and hence the identification of drug resistant *M. tuberculosis* and monitoring of its transmission are critical for the control of the disease. Thus, the drug sensitivity profiles of *M. tuberculosis* isolated from study site were evaluated. In addition, it has been well established that TB and its response to treatment are affected by different factors related to the host and the environment. Co-infections with parasites and undernutrition are important factors, which can affect the progression of tuberculosis and its response to treatment. Thus, it is important to assess the presence of parasitic co-infections and malnutrition in the epidemiological studies of TB. Such studies would lead to the appropriate treatment of parasites and supplementation of nutrition for the tuberculosis patients during anti-TB therapy.

3.1. Research Hypothesis

1. The study area is relatively dispersedly populated zones so that relatively large numbers of strains of *M. tuberculosis* are circulating within the population. Thus, the diversity of the strains of *M. tuberculosis* in the study area is relatively high. In addition, because of the geographic proximity of the present study area with Weldiya from where the Lineage 7 was identified earlier, good number of isolates from this study could belong to the Lineage 7.
2. The magnitude of drug resistance in the isolates of *M. tuberculosis* circulating in the study could be relatively higher than the national average percentage because of repeated treatment and misuse of anti-TB drugs.
3. The magnitude of undernutrition is significantly high in the study area, Oromia Special Zone and South Wollo Zone of the Amhara Regional State due to frequent hit by a drought.
4. The prevalence TB and parasitosis co-infection is relatively high because of the conducive environment for the parasites and their vectors.

3.2. Objectives

3.2.1. General objective

The general objective of this study was to investigate molecular epidemiology, drug sensitivity profiles and associated risk factors of tuberculosis in selected foci in northeastern Ethiopia.

3.2.2. Specific objectives

1. To investigate the molecular epidemiology of tuberculosis in the South Wollo and Omoia Special Zones of the Amahara Region, Northeastern Ethiopia
2. To investigate the drug sensitivity profiles of *M. tuberculosis* isolated from the study area
3. To assess the magnitude of undernutrition in TB patients recruited from the South Wollo and Omoia Special Zones of the Amahara Region, Northeastern Ethiopia
4. To estimate the magnitude of TB and parasitosis co-infections in the South Wollo and Omoia Special Zones of the Amahara Region, Northeastern Ethiopia

4. Materials and Methods

4.1. Description of the Study Area

The study was conducted in Oromia Special Zone and South Wollo Zone of the Amhara Regional State, northeastern Ethiopia. The area was selected purposely for this study in that there was paucity of information for molecular typing and drug sensitivity profiles of *Mycobacterium tuberculosis.*There were also no reported documents that show the status of TB-parasitic co-infections and the associated factors. Kemise Town is the administrative center for the Oromia Special Zone since 1994 with a latitude of 10°43′27.4“N and longitude of 39°52’24.03”E. The town is found at an altitude of 1447m and a distance of 325 km northeast of Addis Ababa. On the other hand, Dessie Town is the capital of South Wollo Zone having a north latitude and east longitude of 11°8′ and 39°38′, respectively. Dessie has an average altitude of 2475 m a.s.l and located 401 km Northeast of Addis Ababa(Alemayehu *et al*., 2016).

Administratively, the Oromia Special Zone has seven districts. Residents of the Zone receive governmental health service from 27 health centers and one general hospital that partially began its function at Kemise Town since 2015. The two towns administration of Oromia Special Zone (Kemise and Bati) have one governmental health center each and were accessible for pulmonary tuberculosis (PTB) sample collection. Similarly, South Wollo Zone has 18 districts and two town administration (Dessie and Kombolcha) (Figure 2).

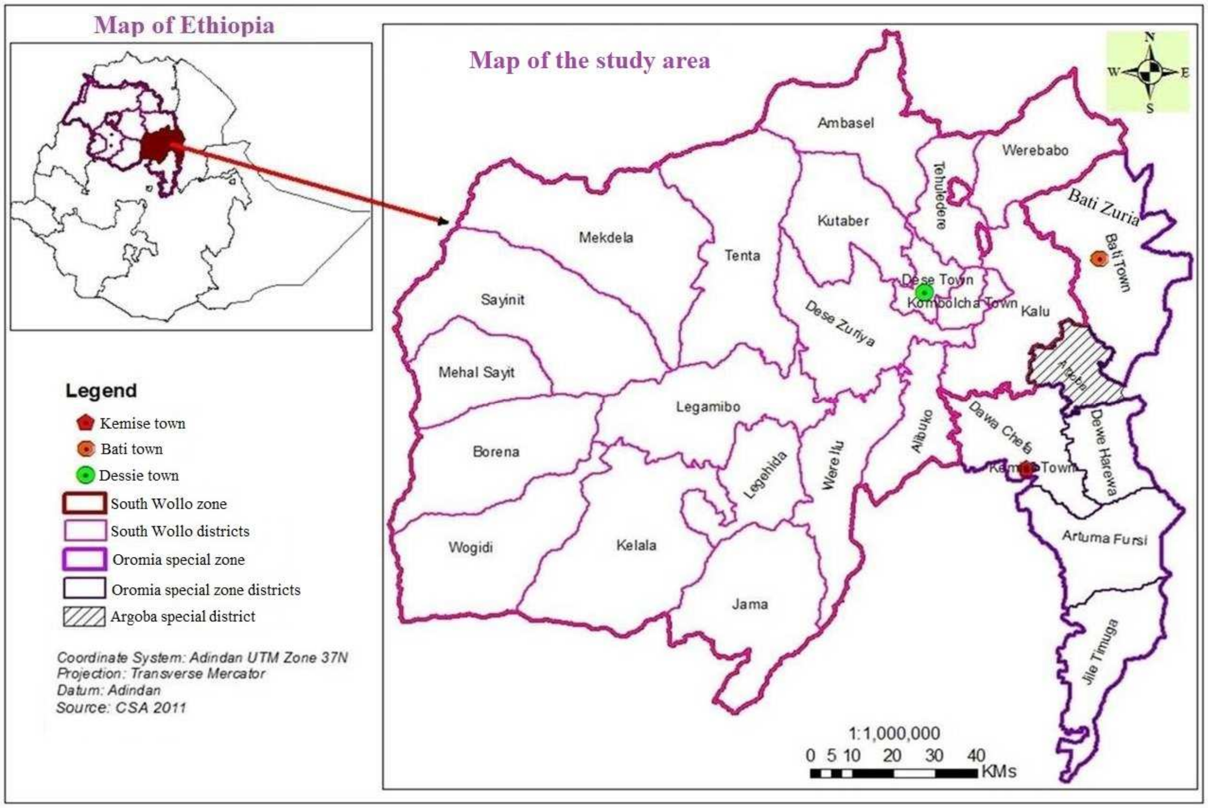


Figure 2. Map of Ethiopia showing the catchment areas of the patients, April 2015 to January 2017

Majority of the inhabitants are Muslims, while minorities are Christians of different denominations. The main occupation of the local society is subsistence agriculture, livestock herding and trade. Indeed, this special Zone has disastrous sites affected by drought and floods with an additional exposure of 100% to malaria. Sometimes, Afar pastoralists move to Chefa valley for the sake of grass for their cattle when there is drought. The societies are with poor hygiene and chew ‘khat’ either separately or in peers. Moreover, a number of people from Amhara, Oromia and Afar meet on a great “Monday market” at Bati town administration causing the highest risk for TB transmission.

Due to few cases of tuberculosis in this Special Zone, the study site was extended to Dessie Town where more samples were expected from Dessie referral hospital (DRH), Biqat higher diagnostic laboratories (BHDL), Dessie health center (DHC) and Boru meda hospital (BMH) that are found in Dessie Town. In addition to sputum smear test, a molecular diagnostic GeneXpert was used for TB diagnosis at DRH and TB suspected FNA samples were collected at BHDL. Both DRH and BHDL serve as referral centers for patients coming from other general hospitals, medical centers and private health institutes of mostly Oromia Special Zone and South Wollo.

4.2. Study Population and Sample Size Estimation

Tuberculosis cases confirmed by the health personnel (pathologist, medical doctors, health officers, nurses and laboratory technologists) and those who fulfilled the inclusion criteria were included in the study. The samples (sputum, Fine-needle aspirates (FNAs) and stools) were collected on the spot from consenting participants until the expected sample size was achieved. In addition, non-active TB cases using clinical symptoms (cough, sputum production, haemoptysis, dyspnea, anorexia, chest pain, fever, drenching night sweat, weight loss and weakness), apparently healthy individuals matched with TB cases by age (with a difference of 5 year), sex and place of living were used as control to compare the nutritional status. These controls were not bacteriologically and parasitologically confirmed for TB and parasitosis, respectively. On the other hand, those individuals who came to the health facilities with the TB cases but didn't fulfill the indicated criteria were excluded.

Preliminary survey was made for 3 months at all governmental health facilities of the seven districts in Oromia Special Zone. Based on the availability of samples (convenience sampling) and transportation access, the data were collected from Kemise and Bati Towns health centers of the Special Zone. In addition, to get more TB cases Dessie Referral Hospital (DRH), Biqat Higher Diagnostic Laboratories (BHDL), Dessie Health Center (DHC) and Boru Meda Hospital (BMH) were also the sample collection sites from Dessie Town. Clinically suspected and bacteriologically confirmed patients using sputum smear test in all study sites were included in the study. GeneXpert was additionally done for TB diagnosis of some suspected cases at DRH. For EPTB, the suspected samples were collected and examined at BHDL by experienced pathologist.

For determination of sample size, co-infection prevalence (33.3%) of *M. tb* and intestinal parasitic infections (IPIs) in Northwest Ethiopia (Alemayehu *et al*., 2014), 95% confidence in the estimate and 5% margin of error were considered. This resulted in a sample size of 341 smear positive TB cases. To compensate for the non stool providing TB cases, inadequate sputum specimen for culture and to have better coverage of the study population an additional 12.6% of the minimum sample size was considered and the final sample size was calculated as 384.

4.3. Inclusion and Exclusion Criteria

Tuberculosis cases from April 2015 to January 2017 who were 18 years and older and willing to participate in the study based on their written voluntary consent were included. This is due to higher proportion of TB cases at the indicated age. Those with severe TB and unable to provide sputum, and those who gave their sputum for TB examination but couldn't provide faecal specimens were excluded from the study.

4.4. Study Design and Laboratory Processing

Purposive sampling technique of an institution based cross-sectional study design was employed targeting to all forms of TB cases. Dry, translucent, leak-proof 50ml capacities of falcon tubes were used to collect a minimum of 3-5ml sputum sample from BMH where drug resistant TB cases were handled and treated. Likewise, a 30ml sputum cup was used for sputum collection from the rest of the study sites. The samples were labeled using indelible labeling marker. Disposable gloves and respiratory masks were used when samples were collected from suspected patients. The sputum was collected from all those smear-positive TB confirmed participants of both previously untreated (new) and previously-treated (retreatment) cases from April 2015 to January 2017. For all study participants, information on the socio-demographic data (sex, age, educational level, residence and chewing of ‘Khat’), history of previous TB treatment and drug susceptibility profiles were recorded on the spot of data collection.

Microscopy and GeneXpert was used at the study sites to screen positive cases. Anthropometric measurements of both BMI and MUAC were made for nutritional assessment among TB positive participants. Similarly, stool examination was made for intestinal parasitic co-infection with an overall study algorithm as in Figure 3.

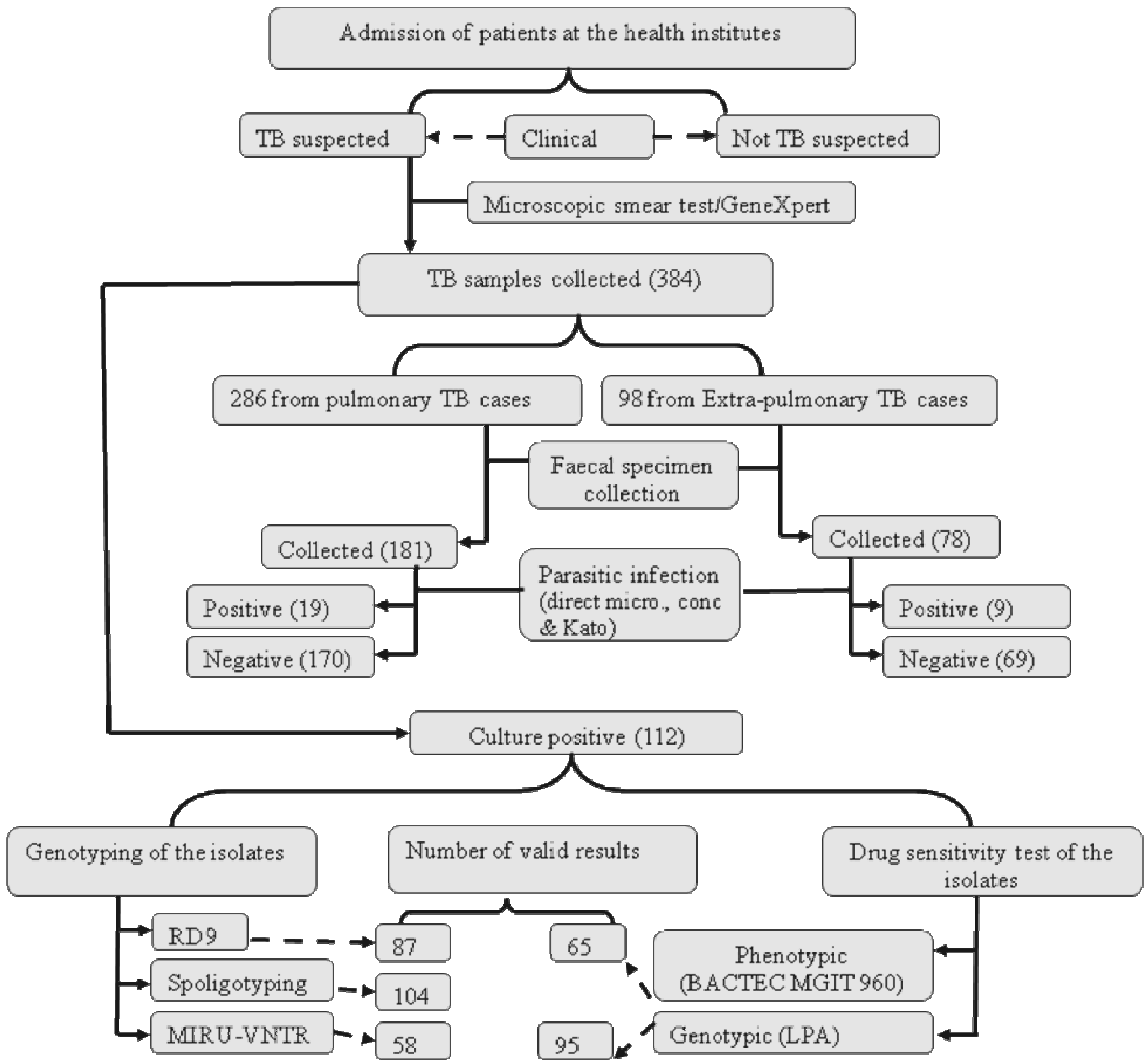


Figure 3. Overall algorithm of the study protocol

4.4.1. Socio-demographic characteristics of the TB patients

A questionnaire having structured and open-ended questions was designed in order to collect data on socio-demographic characteristics and assessment of knowledge, attitude and practice about the cause, mode of transmission and preventive methods of tuberculosis. In addition, issues like educational level, occupation, known contact of TB patients and prior *M. tb* infection were assessed. The questionnaire was prepared in English and then translated into the local languages (Amharic and Afaan Oromo) by an expert who was fluent in both languages to maintain its consistency. It was filled independently by health professionals at the time when the respondents were not perfect enough to read and write.

4.4.2. Bacteriological examinations

a. Ziehl-Neelsen staining

Direct microscopic examination of the sputum for AFB after Ziehl-Neelsen staining technique was performed at sample collection sites (health institutes). For this, a portion of the specimens was air dried and fixed. The fixed smear specimens were stained using Ziehl- Neelsen (ZN) staining techniques (Appendix IV) and examined under oil immersion magnification. The results were identified as positive or negative for AFB and graded based on the bacterial load in the sputum smears. For such grading scales by using florescent microscope at a magnification of 200X, it was considered as negative when no AFB/in at least 30 fields, scanty (1-29 AFB/30 fields), 1+ (30-299 AFB/30 fields), 2++ (10- 99 AFB/ in at least 15 fields) and 3 +++ (> 100 AFB/field in at least 6 fields).The specimens taken from positive patients were kept at a range of -10 to -20 ^{0}C in the refrigerator until being transported to Aklilu Lemma Institute of Pathobiology (ALIPB) for culturing following the protocol described elsewhere (WHO, 2010)

FNA samples from patients suspected for EPTB was collected using a 21-gauge needle attached to a 10 ml syringe with maximum care and safety by an experienced pathologist (Birhanu *et al*., 2014). Afterwards, the Ziehl-Neelsen smear technique was performed by the same pathologist to check its positivity. From positive patients of the smear, a suction of about 1 ml samples were collected for this research purpose and preserved in sterile and tightly closed nunc tubes with phosphate buffer saline of 1 ml at pH 7.2 and kept in the same refrigerator (Yang *et al*., 2004; Berg *et al*., 2015). Finally, both sputum and FNA samples were transported from temporary storage site of the health institutions in a cold chain of 4 °C to TB laboratory of ALIPB in Addis Ababa. At ALIPB the samples were kept in a deep freezer of – 80°C until culturing was done (Wright, 2012).

b. Mycobacterial culturing

A stock of selective LJ media with ingredients of L-Asparagine (2.25g/l), Mono-potassium Phosphate (1.5g/l), Magnesium Sulfate (0.15g/l), Magnesium Citrate (0.375g/l), Potato Flour (18.75) and Malachite Green (0.25g/l) was used for the preparation of *Mycobacterium* culture media. The L-Asparagine and Potato Flour are sources of nitrogen and vitamins, respectively. Mono-potassium Phosphateand Magnesium Sulfate enhance growth of the organism and act as a buffer. Malachite green prevents the growth of many contaminants but encouraging the growth of Mycobacteria. Of this stock, 37.2g was suspended in 600ml distilled water and 12 ml of glycerol was added. Then frequent agitation was made for a minute by boiling. In the case of *M. bovis* cultivation, the glycerol was replaced by 10g of sodium pyruvate and similarly prepared.The glycerol serves as a carbon source and is suspected as favorable for the growth of the human type tubercle bacillus while being unfavorable to the bovine type. The final solution was cooled and sterilized in autoclave at 121^0C for 15 minutes.

For the preparation of homogenized whole eggs, fresh hen eggs were washed with running water and sterilized by 70% ethanol before breaking to mix by shaking under aseptic condition. One liter of this homogenized egg was added to the prepared autoclaved media solution, shacked and sieved with sterile sieve. This egg suspension provides fatty acids and protein required for the metabolism of *Mycobacteria*. When heated, the egg albumin coagulates providing a solid surface for inoculation. The completely prepared egg medium of 6-8ml volume was dispensed into screw-capped culture tubes and their lids were securely fastened. Finally, the tubes were slanted at 15-20^0 angles in inspissator and heated at 85 ^{0}C to solidify/coagulate the media for 45 minutes. On the other hand, at the time when there was a shortage of a stock LJ media, the indicated mineral salt ingredients were measured and used for the preparation of LJ medium. In both cases, the prepared media was left for 48 hours before culturing the bacteria to test their contamination and those poor quality media were identified and discarded.

Mycobacterium culture was done following Tripathi *et al*. (2014) procedures. In the institute's TB culture laboratory, the *M. tb* specimens were digested and decontaminated using Petroff's method. For this, equal amount of 4% NaOH was added to the sputum in 15ml screw-capped falcon tube and shacked to be homogenized. It was followed by centrifugation at 3,000rpm for 15 minutes at room temperature (22^{0}C). Most of the supernatant portion was discarded and the remaining (approximately 500µl) was re-suspended with the pellet. A single drop of phenol red indicator was added to the sediment and neutralized by drops of 2N HCL. Neutralization was considered when the color of the solution was changed from purple to yellow and cultured on two LJ slant media one containing 0.75% glycerol and the other as 0.6% pyruvate. Likewise, for EPTB the samples were mixed thoroughly using sterile injection needle after being thowed and 3-5 drops of it was directly sucked from the nunc tube using the needle and inoculated to the two LJ medium.

Finally, all inoculated LJ slants in culture tubes were incubated aerobically at 37 ^{0}C in a slanted position and contamination was also checked daily for the first week. The inoculated media was positioned in an upright position starting from the second week and colony formation (growth) of the bacteria was observed every week for eight consecutive weeks. Growth was usually suspected at week three and if there was no any form of colony formation till the eighth week, it was regarded as negative culture and the sample was safely disposed. Growth of the *Mycobacterium* was verified by detection of its typical colony morphology and for weakly grown colonies, sub-culturing was done in a new media.

Approximately two loops full of cells from the culture grown colonies were taken and mixed in 200 µl dH2O to be heat killed and extract the DNA for molecular analysis. Heat killing was done by placing the cryotubes containing the isolates to a water bath of 80^0 C for 1 hour. On the other hand, similar amount of culture grown colony was placed in 1ml of the freezing media (Appendix IV). In both cases the isolates were kept in a deep freezer of -80^0C until molecular characterization was done using region of differentiation, spoligotyping and MIRU-VNTR.

4.4.3. Molecular typing

Molecular identification of the MTBC isolates to the species level was done by using deletion typing of region of differentiation. Spoligotyping and MIRU-VNTR 24-loci patterns were used for further characterization of the strains into main phylogenetic lineages, sub-lineages and also perform cluster analysis based on the best match of the reference data base available online. The region of differentiation (RD9), spoligotyping and 24-loci MIRU-VNTR typing were determined based on the presence or absence of *Mycobacteriumtuberculosis* complex chromosomal region of difference(RD) deletion locus, spacers and number of allelic repeats at each locus, respectively.

a) Differentiation of the *Mycobacterium tuberculosis* complex using region of differentiation (RD9)

For differentiation of the causative agent as *M. tb*, *M. bovis* or other forms of MTBC species among TB cases region of difference was used. In this study RD9 was implemented to differentiate *M. tuberculosis* from other species of MTBC. Presence of RD9 revealed the causative species as *M. tuberculosis* andits absence showed the causative agent is not *M. tb* but might be other forms of MTBC or the DNA is missed from the sample (Rajender *et al*., 2005).

Genomes of the isolates were amplified by PCR to detect the presence or absence of RD9 region. This typing of the *Mycobacterium* was done using heat killed cells and the genomes oligonucleotide primers each at a concentration of 100 mM. Those oligonucleotide primers used to notice the presence or absences of RD9 PCR in the study were RD9 FW (5′-AAC ACG GTC ACG TTG TCG TG-3′), RD9 INTREV (5′-TTG CTT CCC CGG TTC GTC TG-3′) and RD9 REV (5′-CAA ACC AGC AGC TGT CGT TG-3′). For preparation of the reaction mixture; 10 µl of HotStarTaq Master Mix (Qiaged, Crawley, UK), 7.1 µl distilled water and 0.3 µl of each primers were used. Each of this reaction mixture was multiplied by the number of samples to be analyzed to prepare the total amount of mixtures required. 18µl of the Master Mix was taken and mixed with 2 µl DNA template (heat killed cells) in a PCR tube giving a total volume of 20 µl. Finally, amplification was processed on each of the samples using a thermocycler for typing purpose. The PCR reaction mixture was heated at 95°C for 15 minutes, after which it was subjected to 35 cycles consisting of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute in the PCR thermocycler (VWR, UK). Then the reaction mixture was maintained at 72°C for 10 minutes.

The resulting PCR products were visualized by standard gel electrophoresis. For this, 0.8 µL of the PCR product was mixed with 0.2 µL of a loading dye and poured into the holes of 1%

agarose gel, after which it was electrophoresed at 110 V and 400 mA for 35 minutes. The gel was visualized by using a UVP photodoc imaging system. Photograph was taken by a photo-camera and interpretation of the result was based on the detection of the bands at different sizes in comparison to positive (*M. tuberculosis* H37Rv and *M. bovis*) and negative (distilled water) controls used (Debebe *et al*., 2014). This RD9 speciation was done to all the culture positive isolates and further characterization was performed by using spoligotyping and MIRU-VNTR to the strain level.

b) Spoligotyping and its result interpretation

Spoligotyping was performed using the spoligotype kit supplier's instructions. It was based on polymorphism of the chromosomal direct repeat (DR) locus that contains a variable number of short direct repeats interspersed with non-repetitive spacers. The DR region was amplified by PCR using oligonucleotide primers derived from the DR sequence. The Primers used in the amplification process were DRa 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated at 5' end) and DRb 5'-CCGAGAGGGGACGGAAAC-3' as originally described by Kamerbeek *et al*. (1997).*M. tuberculosis* H37Rv and *M. bovis* BCG were used as a positive control where as an autoclaved distilled water was used as a negative control in the study.

For the PCR process, 10 µl of Hot Star Taq, a Master Mix of 2 µl from each primer, 5 µl suspension of heat killed cells, and 8 µl distilled water were used. This mixture was heated for 15 min at 96^{O}C, 1 min at 55^{O}C and 35 sec at 72^{O}C. Standard thermo cycler (VWR Thermo cycler, UK) was used for this amplification. The amplified PCR product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus (Appendix V). After hybridization the membrane was washed twice for 10 min in 2 x SSPE and 0.5% sodium dodecyl sulfate at 60^{O}C and then was incubated in 1:4000 diluted streptavidin peroxidase for 60 min at 42^{O}C. The membrane was washed twice for 10 min in 2 x SSPE for 5 min at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence ECL detection liquid followed by exposure to X-ray film as described by the manufacturer (Hain Life Science Company). Finally, for repeated use of the membrane, it was stripped by washing 2x for 30 min each time in 1% SDS at 80°C and then incubated for 15 min in 20 mM EDTA (pH 8) at room temperature. The membrane was then sealed in its plastic container and stored at 4°C for future use.

The autorad spoligotyping results were checked visually by three experienced operators and the spacers were written in binary format using an English small letters 'o' and 'n' when the spacers are absent and present, respectively. The binary representation was converted to the octal code and entered to the international database (SpoIDB4) and SITVIT2 (Institute Pasteur de Guadeloupe) to determine and interpret the specific *M. tb* complex strain and SIT, respectively. The novel isolates which were not so far described in the existing spoligotyping data base profile using SITVIT2 lists were considered as orphans (Streicher *et al*., 2007; Dou *et al*., 2008).

c) MIRU-VNTR typing

MIRU-VNTR genotyping technique for the *Mycobacteriumtuberculosis* complex characterization is not performed in Ethiopia so far. Thus, molecular characterization of the tuberculosis disease causing agent using this technique was done outside the country where the facilities are accessible. The DNA templates or heat killed positive TB bacterial cells were sent abroad (Yimer *et al*., 2015; Ali *et al*., 2016; Bedewi *et al*., 2017; Mekonnen *et al*., 2018). Finding a support from abroad, sample preparation, packaging to the standard, getting permission from exporter and importer countries, and shipping process were challenging. In this study, establishment and optimization of the protocol were performed at ALIPB-AAU with the support of NIH-TB funded research project through H3 Africa. Molecular characterization of the *Mycobacteriumtuberculosis* complex by MIRU-VNTR in the country was done as follows:

PCR amplification procedures for 24-loci MIRU-VNTR

The PCR amplification Master Mix was prepared for 26 PCR tubes of which 24 pairs of the primers (forward and reverse) were for a single isolate. The Master Mix was constituents of 5x buffer (HF), 10mM dNTPs, Phusion DNA polymerase, DNA template and dH_2O as indicated in Table 2 and 24µl of the Master Mix was distributed to each of the respective 24 PCR tubes. Then 1µl of each 24-locus MIRU-VNTR primers were added to the tubes making a final volume of 25µl for the amplification. Of the collected MTBC isolates in the study, 69 sub-culture positive samples using MGIT 960 system were heat killed and used as DNA template in the MIRU-VNTR genotyping.

Table 2. Components and volumes of MIRU-VNTR Master Mix used in the study for each isolate

5 x buffer (HF)	5µl x 26 = 130 µl
10mM dNTPs	2µl x 26 = 52 µl
Phusion DNA polymerase	0.5µl x 26 = 13 µl
DNA template	0.8µl x 26 = 20.8 µl
dH_2O	15.7µl x 26 = 408.2 µl
Total	624 µl

Unlabeled PCR primers were used in the study for the 24-locus set as in Supply *et al*., 2006 (Appendix VI) as a result each loci were amplified independently. PCR program was run with its cycling conditions starting at an enzyme activation step of 15 minutes at 95°C, followed by 40 cycles of 1 minute at 94°C for denaturation, 1 minute at 59°C for annealing, and 1 minute 30 seconds at 72°C for extension. Thereafter, the reactions were incubated for 10 minutes at 72°C for final extension/elongation. A positive control, H37Rv, and a negative control, sterile distilled water, were used in the study and the amplified PCR products were run in a gel-electrophoresis (Cowan *et al*., 2002; supply, 2005).

Agarose Gel-electrophoresis detection

Sixty nine amplified PCR products of *M. tuberculosis* isolates were electrophoresed to determine the size of amplicon. Electrophoresis was made on 1.8% (w/v) 300 ml agarose gel with 15 µl ethidium bromide in 1X Tris Borate-EDTA (TBE) buffer run at 120 volts and 400 milliampere for 5 hours. Briefly, 5.4gm of UltraPure™ Agarose powder (Sigma Life Science) was added to 300ml 1 x TBE buffer and heated in a microwave to dissolve the powder. The TBE buffer was prepared by dissolving 10.8gm tris base and 5.5gm boric acid in 805ml dH_2O and adding 4ml of EDTA pH = 8.0 with a final volume adjusted to 1000 ml by adding more distilled water.

Ethidium bromide was added to the dissolved agarose at 45^0C or when it was possible to hold a boiled agarose gel bottle with a hand and gently homoginized. Finally, it was poured to a gel tray (Thermo Scientific Owl A2 Large Gel System, 20 x 25 cm) inserted with 1 mm width 26 lane gel combs. The comb was removed with care after 45 minutes and 3µl of amplification products mixed with 2.5 µl ofloading dye was added to the wells. DNA ladders of 100 bp and 50 bp molecular standards were included on each of the gel run. The gel tank lid was placed and connected to a power supply unit. Product sizes were visualized and photographed by using an ultraviolet (UV) transilluminator. Product sizes were established by comparing their bands with the standard DNA ladder bands. The PCR products of H37Rv were loaded to ensure accuracy and the PCR products of sterile water was used to control for reagent contamination.

The numbers of tandem repeats or MIRU-VNTR alleles were determined based on the size of bands (PCR fragments) in a gel and inferring it with interpretation tables (Supply *et al*., 2005; Borroni *et al*., 2012). Then the main phylogenetic predictions were facilitated by using MIRU-VNTR-24 loci profiles into freely accessible, on-line strain identification databases of MIRU-VNTR*Plus* (http://www.miru-vntrplus.org). The isolate patterns were used to compare with the reference strains in the database for the assignment of MTBC species, lineages and genotypes. Phylogenetic dendrogram was calculated based on the neighbor-joining (NJ) clustering algorithmsand a minimum spanning tree (MST) analysis was also performed.

4.4.4. Genotypic drug sensitivity test

a) The molecular GeneXpert

This test was conducted in DRH on MTB/RIF test platform (GeneXpert, Cepheid), which integrates sample processing and PCR within a disposable plastic cartridge. In the hospital TB laboratory, a total of 95 sputum samples were subjected to the Xpert test and the test was performed according to the manufacturer's instruction. Briefly, 2 ml of Xpert MTB/RIF sample reagent (provided with the assay) was added to 15 ml Falcon tube containing 1ml of unprocessed sputum sample in a 2:1 ratio. The tube was manually agitated twice during 15-min incubation at room temperature. Of the liquefied sample, 2 ml was transferred to the cartridge and loaded into a module of the GeneXpert machine connected with an automated readout that reports the existence of *M. tb* and its bacterial load as high, medium, intermediate, low, or very low with an additional detection of RIF resistance (Boehme *et al*., 2010).

b) GenoType MTBDR*plus* line probe assay

This assay was used for identification of *M. tuberculosis* complex and its resistance to rifampicin, isoniazid or both. MTBDR*plus* version 2 LPA was used in this study at EPHI.Heat killed DNA extracts of the isolates were used for the process.

Reagent preparation

For preparation of a Master Mix, a separate reagent preparation room was used to be safe from any form of DNase contaminant. For this, the number of specimens to be tested was initially determined and the required controls plus two more extra reagents were considered for volume loss on pipette tips. The amplification mix per sample was prepared by using 10 µl of AM-A (5 µl of buffer, 2 µl of $MgCl_2$, 3 µl of H_2O and 0.2 µl of *Taq* polymerase) and 35 µl of AM-B (Primer Nucleotide Mix - PMN). This volume was taken into account for the preparation of overall Master Mix aliquot and taken to a separate DNA sample addition room.

DNA addition

In the DNA addition room, 45 µl of the aliquot was taken and added into the labeled PCR tube. An adequately mixed 5 µl of the heat killed isolates was also added to each of the PCR tubes (except for the negative control) making a final volume as 50 µl. The overall volume was mixed by gently pipetting up and down via opening one tube at a time. Separate tips and care was given at the time of addition and mixing the samples.

PCR amplification and hybridization

Using a thermal cycler (Serial No.-272S8090145), the following amplification profile (Table 3) was used for denaturing, annealing and extension of the prepared specimens placed in the PCR tubes. Primarily, denaturing was made at 95^0C followed by annealing at lower temperatures and a final extension of the DNAse for 8 minutes at 70^0C.

Table 3. PCR amplification program for GenoType MTBDRplus line probe assay

Time	Temperature	No. of cycle/s
15min	95^0C	1 cycle
30sec	95^0C	
2min	65^0C	10 cycles
25sec	95^0C	
40sec	50^0C	20 cycles
40sec	70^0C	
8min	70^0C	1 cycle

For hybridization, 20 µL of the amplification products were mixed with 20 µL of the denaturing reagent (provided within the kit) and denaturing was performed for 5 minutes at room temperature in each of the plastic tray well. One ml of pre-warmed (45°C) hybridization (HYB) buffer was added into each well and a strip was placed into each well of the tray and mixed carefully. Hybridization was performed at 45°C for 30minutes and the hybridization buffer was removed from the well. One ml of streptavidin was added to each of the tray well with strips and incubated for 15 minutes at 45°C. After removal of the streptavidin, the strips in the well was washed using 1 ml RIN. The RIN was completely removed and streptavidin conjugated with alkaline phosphatase was added for colorimetric detection of hybridized. Two washing step with RIN and one washing step with distilled water was carried out at room temperature. Finally, 1 ml of diluted substrate (10 µL SUB-C and 990 µL SUB-D) was added and washed twice with dH2O. At last, the strips were removed and air dried on absorbent paper for observation of the bands.

Interpretation of the line probe assay results

Presence of *Mycobacterium tuberculosis* complex was determined by looking at TUB band of the strips and identified for drug sensitivity test (4).

Table 4. Interpretation of MTB+ and MTB- in line probe assay

MTB+	RIF-resistance	INH-susceptible
MTB+ (MDR)	RIF-resistance	INH-resistance
MTB+	RIF-susceptible	INH-susceptible
MTB+	RIF-susceptible	INH-resistance
MTB-	Not *Mycobacterium tuberculosis* complex	

Each strip had 27 reaction zones (Figure 4) with six controls as conjugate control (CC), amplification control (AC), *M. tuberculosis* complex control, *rpoB* locus control, *katG* locus control, and *inhA* locus controls. The remaining 21 reaction zones are wild type (WT) and mutation (MUT) reaction zones (eight *rpoB* WT and four *rpoB* MUT probes, one *katG* WT and two *katG* MUT probes, and two *inhA* WT and four *inhA* MUT probes). Presence of CC band was considered to verify the efficiency of conjugate and substrate. Presence of AC band indicates the efficiency of DNA extraction and PCR procedures. Occurrence of MTBC band indicates that the tested bacterium belongs to the *M. tuberculosis* complex. The three respective locus control bands (*rpoB, katG and inhA*) were considered to show the presence of the specific gene region (Yadav *et al*., 2013).

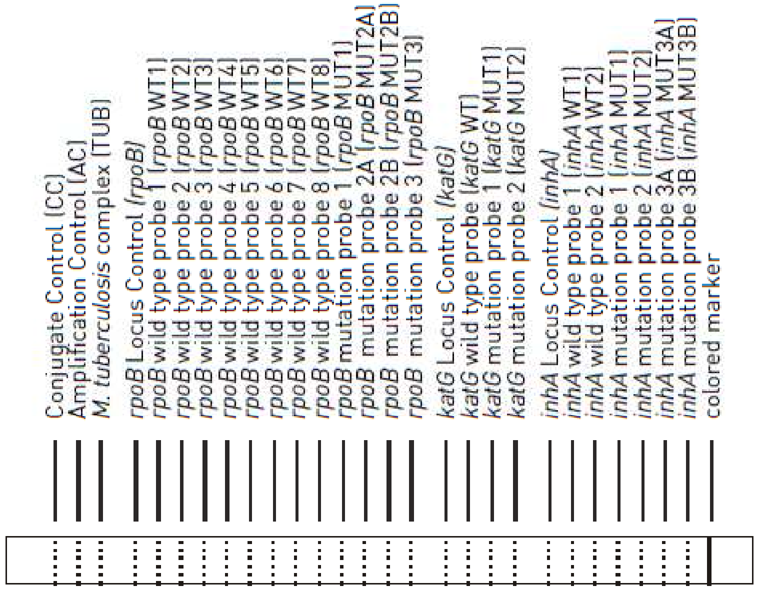


Figure 4. Pattern of GenoType MTBDRplus (LPA) strip with a total of 27 reaction zone (adopted from Yadav *et al*. 2013)

Finally, MTBDR*plus* V2 Instruction Manual was used for interpretation of drug sensitivity test based on manufacturer's guideline (Hain Lifescience, Nehren, Germany) (Figure 5). As it was written, the developed strips were fixed on an evaluation paper provided with the kit by aligning the bands CC and AC with their respective lines on the sheet for interpretation. Development of only CC and AC bands on the strip was considered as a valid negative result. Presence of TUB band indicates that the sample contains a member of *M. tuberculosis* complex. The test was not evaluated when one or both of the locus control bands (*rpoB* and/or *inhA*) were missed. But in rare cases when the *katG* control band was missed, it was considered as the strain tested is INH resistance. Absence of a signal for at least one of the wild type probe implied resistance to the respective antibiotics. Missing of bands in *rpoB* probes showed resistance to rifampicin whereas missing of *katG* and/or *inhA* indicates INH resistance. On the other hand, the mutation probes were also considered as resistant when the bands are as strong as or stronger than the existing AC bands.

Absence of the WT band is usually accompanied by the presence of MUT, which indicates resistance. Presence of all WT bands without the MUT band was interpreted as susceptible isolate. In rare cases, missing of WT band(s) without a corresponding MUT band might be observed which was considered as due to "uncommon mutations" in the probe region. Presence of both WT and MUT bands in the same stripe might be an indication for the presence of hetero-resistance or mixed infection.

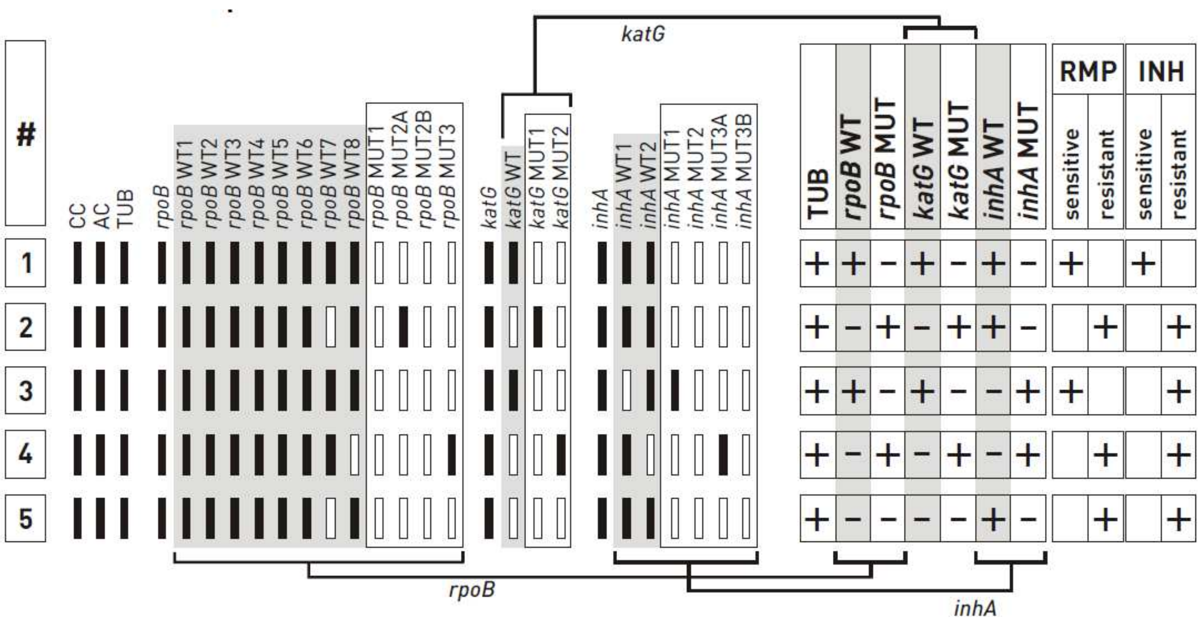


Figure 5. Interpretation of banding patterns and their evaluation with respect to RMP and/or INH resistance (Adopted from MTBDRplus V2 Instructions for use IFU-304A-02)

c) Phenotypic BD BACTEC ™ MGIT ™ 960 System

Phenotypic DSTs were conducted using BACTEC™ MGIT™ 960 SIRE Kit (Becton Dickinson, Baltimore, MD, USA). Sub-culturing of 112 LJ-culture positive freezed isolates was done using the MGIT 960 instrument. The drug sensitivity test to streptomycin, isoniazid, rifampicin and ethambutol was performed for all of the successfully recovered sub-culture positive isolates in the study.

Sub-culturing of the isolates

Sub-culturing was made from the freezed samples using the reagents MGIT medium (7ml in the MGIT tube), MGIT growth supplement (enrichment) and MGIT PANTA™ (Polymixin, Amphotericin B, Naladixic acid, Trimethoprim and Azlocillin) to suppress contamination. The PANTA was reconstituted with 15.0 ml MGIT growth supplement and mixed until completely dissolved. 0.8 ml of the mixed enrichment was taken and poured into each MGIT tube containing 7ml of the MGIT medium. Finally, 0.5 ml of the TB isolates was added and loaded into the MGIT instrument. Shaking the contents of MGIT tube was not done to maintain the oxygen gradient in the medium. The tubes were simply recorded by the BACTEC MGIT 960 instrument and placed in the machine at the incubation temperature of 37 $\pm$ 1^0C. The sub-cultured samples were kept in the instrument until it flags them as positive.

The *Mycobacterium* growth was detected as granular while contaminated bacterial growth appeared as extremely turbid. Such growth of the *M. tuberculosis* complex is also settled at the bottom of the tube. Removal of the sample was done when green light is observed at the tubes

exact location in the drawer of the instrument. At the end, the remaining samples were flagged out as negative (no growth) after a maximum of 42 days and the results in all cases were printed out by printer connected to the MGIT instrument (Siddiqi *et al*., 2012).

Work-up on positive sub-culture results

For BACTEC MGIT 960 SIRE kit susceptibility test, five 7ml MGIT tubes were labeled for each test isolates. One was labeled as GC (Growth control), one as STR, one as INH, one as RIF and the remaining as EMB. To each of the tubes 0.8ml of BACTEC MGIT SIRE supplement from the kit was added. A final concentration of 1.0µg/mL (STR), 0.1µg/mL (INH), 1.0µg/mL (RIF) and 5.0µg/mL (EMB) were also used in the corresponding tube (Table 5). No antibiotic was added to the GC tube. About 0.5mL of the organism suspension was also added to all the five MGIT tubes. The tubes were tightly recapped and mixed thoroughly by gentle inversion three to four times before entering into BACTEC MGIT instrument. In the instrument, the result was continually monitored until a susceptible or resistant result was reported by the instrument (Aktas *et al.*, 2014).

Table 5. Drug concentration and volumes in MGIT tubes for SIRE drugs

Drug	Concentration of Drug after Reconstitution*	Volumes 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 were reconstituted using 4 ml of sterile water to achieve the concentrations indicated (Becton DickinsonBACTEC MGIT 960 SIRE Kits manual)

Interpretation of BACTEC MGIT 960 results

When the growth unit (GU) of the GC reached at a value of 400 or more, the instrument that showed susceptibility test was completed and removed after scanning with a printout of an inventory report. Susceptibility results for both INH and RIF (in the first option) were interpreted by the instrument as “S” or “R” for susceptible and resistant samples, respectively. At the time the GU value of the GC was 400 or more and if the GU value of the drug tube was less than 100, the test result was reported as “susceptible,” while if the GU value of the drug tube was 100 or more the result was interpreted as “resistant.” The GU values of both the DST sets were retrieved and recorded. In case the GU value of the control did not reach 400 within 21 days, the instrument indicated an X200 error, indicating insufficient growth. On the other hand, if the GU reached 400 earlier than day 4, the instrument gave an X400 error, indicating contamination or overinoculation (Siddiqi *et al*., 2012).

4.4.5. Assessment of nutritional status

Age, sex, body mass index (BMI), Mid-upper Arm Circumference (MUAC) and other clinical symptoms like coughing for ≥ 2 weeks, night sweating, body temperature (fever), loss of appetite and general body conditions of the cases were measured and recorded. The BMI of participants was calculated by measuring individual's weight in kilograms and height in meters using the formula:

$$BMI = \frac{mass(kg)}{(height(m))^2}$$

Measurement for determining BMI was done by ultrasonic height and weight machine at BHDL. The print outs of the machine were collected and registered for each of the cases. This measurements was used to assess how many of the individual's body weight with an infection (TB cases and TB parasitic co-infection) departs from what is normal or desirable for a person of his or her height which might be due to TB infection, parasitic infections or co-infections.

Meanwhile, the risk of developing TB and malnutrition was determined using both BMI and MUAC parameters.

The BMI value was categorized as underweight (BMI $\leq$ 18.5), normal (18.5 < BMI $\leq$ 25), overweight (25 < BMI $\leq$ 30), and obese (BMI >30). Weighing of the participants was made to the nearest 0.1kg and the proportion of mildly (BMI 17.0-18.4kg/m^2), moderately (16.0kg/m^2-16.9kg/m^2) and severely (BMI <16.0kg/m^2) malnourished participants were identified. Measurement of their height was carried out without wearing shoes, to the nearest 0.1cm and all values were recorded (WHO, 1995; Hanrahan *et al*., 2010).

Arm circumference measurements were computed with the subject standing upright, shoulders relaxed, and the right arm hanging loosely. It was considered that muscle of the arm is not flexed or tightened, which could yield a larger and inaccurate reading. The participant was standing facing his/her right side to the physician and placing MUAC measuring tape around the upper arm at the crossed point (+), perpendicular to the long axis of the upper arm. Holding the measuring tape gently on the skin surface (not compressing the skin) and the tape was rounded to the arm for measuring. The arm circumference measurements for all subjects were also recorded to the nearest 0.1cm (James *et al*., 1994). Based on cutoff values for MUAC by Abrhame and Haidar (2014), the study participants were categorized as undernourished when MUAC is $\leq$ 23cm for males and $\leq$ 22cm for females.

4.4.6. Examination of tuberculosis patients for co-infection with intestinal parasites

Direct wet mount microscopic examination and Kato-Katz techniques were done at the health institutes and examined by laboratory technicians. Intensity of the parasitic infection was determined by counting the number of eggs per gram of feces in the Kato-Katz fecal thick-smear technique following WHO (2011) guidelines. In addition, Sodium acetate-acetic acid-formalin solution (SAF) concentration technique was used for all voluntary TB cases to give up their fecal specimens.

Fecal specimens were collected using stool cup with a tight fitting lid and two applicator sticks given to the TB patients to bring a sizable stool sample of his/her own. The slides were labeled with identification number and a drop of saline (0.9%) was placed at the middle for direct wet mount fecal examination. A small proportion (about a match stick head size) of the specimen was picked up and mixed with the saline and covered with cover slip for microscopic observation under low power objective. The entire cover slip area was systematically examined using 10X objective and when organisms or suspicious material was seen the nosepiece of a microscope switched to high power objective (40X) for observation of the detailed morphology.

A portion of the fresh stool samples (about 1gm) was further preserved in 10 ml SAF solution and transported to ALIPB Medical Parasitology Laboratory for processing using saline ether concentration techniques. The method was used to report different intestinal parasitic infections specifically for the detection of hookworm that cannot be seen by Kato-Katz method since hookworm eggs disintegrate after 30 minutes of specimen collection.

4.5. Quality controls

Positive Acid-Fast Bacilli samples were reserved as a reference standard at the health institutes for quality control of microscopic smear test. The overall laboratory activities were also checked for its accurate diagnosis by regional TB laboratory experts every three month as an external quality control. Color of the LJ-culture media was checked before using and only those with fitting color (light green) without any crack were selected. Prior to the inoculation, all the prepared media were incubated at 37˚C for 48 hours to check any form of contamination. For molecular characterization of the *Mycobacterium* H37Rv, *M. bovis* and distilled water were used in each test as a positive and negative quality control. For drug sensitivity test, a susceptible strain ofH37Rv reference strain (ATCC 27294) was used at EPHI as a positive quality control in all the methods and was tested with each batch of DST. In addition, nuclease-free distilled water was also included as a negative control for each run to detect possible contamination events.

The GeneXpert MTB/RIF assay is an entirely self-contained test with quality control of the various steps included; so it was not necessary to perform testing of quality controls with every batch of tested specimens. In fact, laboratory monitoring and supervision visits were done by the National Tuberculosis Control programme to assess the exact Xpert MTB/RIF test implementation. Modules of the Xpert were also annually checked and calibrated by an external quality control. In addition, internal quality control program with positive and negative controls were implemented during the study.

In order to validate the correct performance of the test and proper functioning of kit constituents for LPA, each strip includes five control zones. A CC zone was used to check the binding of conjugate on the strip and a correct chromogenic reaction. The AC zone was used to check for a susceptible amplification reaction. The three locus control zones (*rpoB*, *katG* and *inhA*) were also used for checking the optimal sensitivity of the reaction for each of the tested gene loci. All materials (such as pipette tips) coming in contact with the reagents were checked as free from DNases. The respective test strips for the negative control showed bands only on CC and AC while in the case of positive control the bands should exist on CC, AC, *TUB*, *rpoB*, *rpoB* WT, *katG*, *katG* WT, *inhA* and *inhA* WT as our quality control for the LPA test. For MGIT's quality control, each batch of new drug was performed with the reference strain which was susceptible to all standard anti-tuberculosis drugs. Training was given for data collectors and supervisors.

To data obtained through questionnaire, pre-testing of the questionnaire was made on 30 study participants for its clarity and appropriateness. Data collection process was strictly followed daily by the supervisor and the principal investigator. In addition to the trainings on BMI measurements, the instruments were also calibrated for the research work.

4.6. Data Analysis

The recorded data was checked for completeness and consistency, and then entered into Microsoft Excel 2007 spreadsheets. The data was then exported to IBM SPSS Statistics for Windows, Version 25.0. (Armonk, NY: IBM Corp., USA) program for analysis.Descriptive statistics was used to determine frequency and percentage. In spoligotyping, the reference data base (SpolDB4) available online through http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/was used to assign the shared international spoligotype numbers (SIT) to known profiles and if not available the new patterns were considered as 'orphan' types. An online tool Run TB-Lineage with a website of http://tbinsight.cs.rpi.edu/run_tb_lineage.html was also used to identify family/clade, lineages and sublineages of the isolates. Those identical spoligotypes patterns with identical DNA genotypes were considered as a cluster and these clustered strains were identified as an indicator for the recent transmission.For recent transmission index (RTI), cluster analysis was calculated using the formula (nc – c)/n, where nc is the total number of clustered patients, cis the number of clusters, and nas the total number of patients in the sample.

The discriminatory power of each locus was evaluated using the Hunter and Gaston Discriminatory Index (HGDI) which was calculated using the equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where; *D* is the index of discriminatory power (0.000-1.000), *N* is the total number of strains in the sample population, S the total number of types described, and *xj* the number of strains belonging to the *j*th type. A *D* value of 1.000 indicates that a typing method was able to distinguish every member of a strain population from all other members of that population. Conversely, an index of 0.000 would indicate that all members of a strain population were of an indistinguishable type. An index of 0.500 would mean that if one strain was chosen at random from a strain population, then there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first (Bikandi *et al.*, 2004). The HGDI value was calculated using the formula manually and checked by discriminatory power calculator online.

Sensitivity, specificity, positive and negative predictive values were calculated to compare the performances between drug sensitivity tests. Agreement between the tests was assessed by Cohen's Kappa statistics. The Kappa value was interpreted with values < 0 as indicating no agreement and 0–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1 as almost perfect agreement (McHugh, 2012). The Pearson Chi-square ($\chi 2$) was used to determine the association of drug sensitivity test and TB type, lineages, dominant strains and clustering of the isolates. Association of TB-parasitosis co-infection and malnutrition with gender and age was also analyzed using chi-square test. The results were considered as statistically significant when the p-value was less than 5%.

4.7. Ethical Considerations

The study was carried out after obtaining ethical clearance from the Institute Review Board of College of Natural and Computational Sciences, Addis Ababa University (Ref. No. CNSDO/392/07/15) but later renewed in July, 2019 (CNSDO/668/11/2019). Letter of permission was also obtained from the health bureaus of the study sites. Prior to conducting the study, objectives of the study were clarified to the study participants and written informed consent was obtained. Subject confidentiality, any special data security requirements were maintained and ensured to the participants. Positive TB case participants were referred to the DOTs program and for possible treatment and follow up.

Laboratory results feedback

Positive results of the sputum microscopy, FNA smear test and LJ-culture results were reported on time to the concerned health institutes for strict follow up and medications. Similarly, positive parasitic infections were reported and medicated by the health institutes during their TB treatment follow up.

5. Results

A total of 384 TB cases (213 males and 171 females) were involved in the study. Tuberculosis and parasitosis co-infection prevalence was 10.8% with intestinal helminth co-infection at greater proportion than intestinal protozoa. The prevalence of undernutrition was 58.6% as determined using body mass index and 73.0% as determined using mid-upper arm circumference with no significant association with gender ($P > 0.05$).

In these study, both forms of TB were identified with pulmonary TB cases as the predominant ones than the extra-pulmonary TB cases. TB lymphadenitis was found to be the most prevalent (85.9%) form of extra-pulmonary tuberculosis with cervical adenopathy (75.3%) being the commonly existing disease. A diverse spoligotyping patterns were identified where most of them (86.5%) were not registered in the global spoligotyping data base. Low proportion of the isolates (20.2%) was also recognized as in clustered forms by spoligotyping. From the study, both modern and ancient Lineages were identified with the modern Euro-American Lineage as the predominant ones. The 24-loci MIRU-VNTR showed all the isolates were orphan and the technique has also the highest discriminatory power of identifying the patterns.

Both MTBDR*plus* assay and MGIT drug sensitivity testing found a high but comparable proportion of resistance 16.8% and 15.9%, respectively. Drug resistance was greater among new TB cases than the retreatment ones. Rifampicin mono-resistance was also in large proportion. A fair agreement was found between the two testing methods (GenoTypic MTBDR*plus* assay and the conventional MGIT test) in detecting *Mycobacterium tuberculosis* with sensitivity, specificity, positive and negative predictive value of 94.2%, 30.2%, 68.4% and 76.5%, respectively.

5.1. Demographic Characteristics of the Study Patients

Most of the cases were recruited from South Wollo Zone (n = 247) and the remaining subjects from Oromia Special Zone. Of the identified TB cases, 369 (96%) were recorded at the district level. It was found out that a quarter of these cases were from a single district (Dessie town) whereas the remaining were recruited from other districts of both South Wollo and Oromia Special Zone of the study site. In addition, 58 (15%) of the cases were from other health institutes of the neighboring Zones (mainly North Wollo zone) referred to as DRH and BHDL (Table 6).

Table 6. Number of smear (GeneXpert) positive and their culture positivity from different districts of the study area, April 2015 to January 2017

Zone	District	Smear (GeneXpert) Positive (%)	Culture Positive (%)
Oromia Special Zone (Total cases = 79)	Bati town	27 (7.0)	9 (8.0)
	Bati district	20 (5.2)	12 (10.7)
	Dawa Chefa	7 (1.8)	4 (3.6)
	Kamise town	19 (4.9)	10 (8.9)
	Artuma Fursi	4 (1.0)	2 (1.8)
	Jile Timuga	2 (0.5)	0 (0.0)
South Wollo (Total cases = 247)	Albuko	2 (0.5)	1 (0.9)
	Ambasel	12 (3.1)	2 (1.8)
	Borena	3 (0.8)	0 (0.0)
	Dessie town	92 (24.0)	27 (24.1)
	Dessie zuria	26 (6.8)	9 (8.0)
	Jama	10 (2.6)	4 (3.6)
	Kallu	24 (6.3)	7 (6.3)
	Kelala	4 (1.0)	0 (0.0)
	Kombolcha	25 (6.5)	8 (7.1)
	Kutaber	7 (1.8)	0 (0.0)
	Legahida	3 (0.8)	0 (0.0)
	Legambo	5 (1.3)	0 (0.0)
	Mekdela	7 (1.8)	2 (1.8)
	Sayint	4 (1.0)	1 (0.9)
	Tehuleder	2 (0.5)	0 (0.0)
	Tenta	4 (1.0)	1 (0.9)
	Wegidi	3 (0.8)	2 (1.8)
	Werebabu	6 (1.6)	1 (0.9)
	Wereilu	8 (2.1)	2 (1.8)
From other zones (43) and non-documented (15)		58 (15.1)	8 (7.1)
	Total	**384 (100)**	**112 (100)**

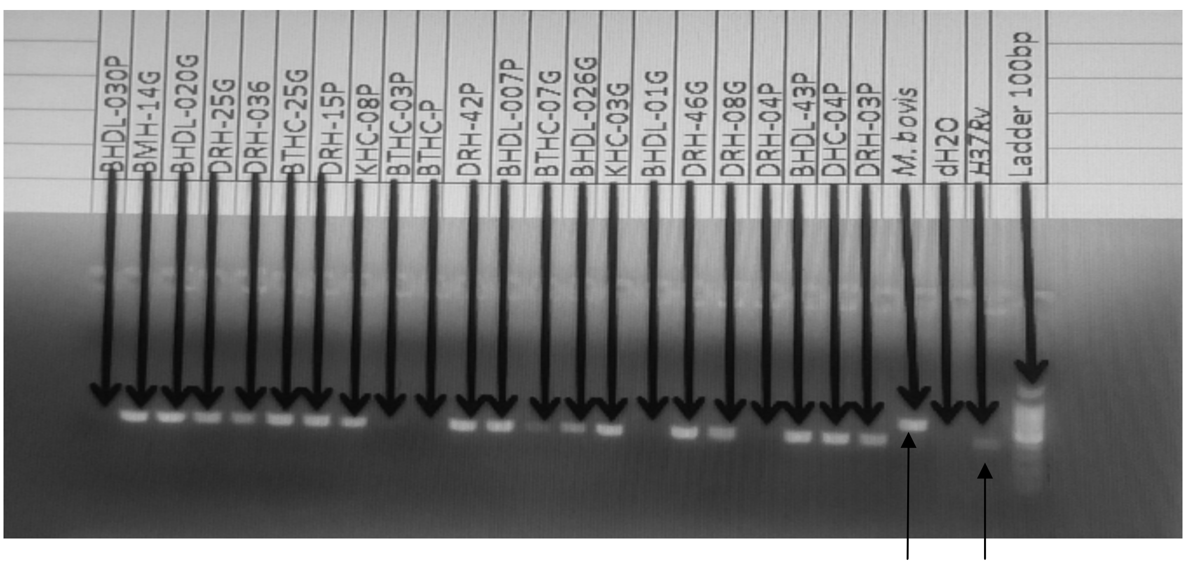
The age range of TB cases were from 18 to 75 years with a mean age of 33.7 (SD = 12) having no significant difference between males (mean = 34.5, SD = 12.3) and females (mean = 32.7, SD = 11.4). The average household size of the TB cases was 4.4. Pulmonary TB cases accounted for

74.5% (286/384), the overall prevalence of TB was highest (67.0%) in the 18–37 years age group and those with family size ranging from 3 to 5 were more affected.

5.2. Molecular Epidemiology of Tuberculosis

5.2.1. Genomic deletion analysis using region of differentiation

Deletion typing via region of differentiation was made in all 112 culture positive isolates and of these 77.7% (87/112) isolates had an intact to the RD9 (396 bp) and identified as *M. tuberculosis*. On the contrary, an interpretable signal was not detected for this region of differentiation in the remaining 22.3% (25/112) isolates. As shown in Figure 6 of the representative agarose gel result RD9 band was not detected in dH_2O (negative control) and five of the study isolates.



575bp 396bp

Figure 6. Polymerase chain reaction (PCR) products of gel-electrophoresis using RD9 primers at ALIPB for some of the study isolates

Lane 1: 100bp ladder; Lane 2: *M. tuberculosis* (positive control); Lane 3: molecular grade H_2O (negative control); Lane 4: *M. bovis* (positive control); lane 5-26: heat killed PCR products of *M. tuberculosis*

5.2.2. Spoligotype results

Spoligotyping was performed on 112 culture positive heat killed *Mycobacterium* isolates. Of these, a total of 92.9% (104/112) gave interpretable results on autorad readings for the formation of spoligotyping patterns (Figure 7). Ten clusters were identified in 20.2% (21/104) of the isolates while the remaining isolates were unique (singletons). Nine of the clusters had 2 isolates each while the remaining one cluster had 3 isolates with similar patterns. In these finding, majority of the patients had unrelated spoligotypes and with different strains of *M. tuberculosis* that acquired the disease from unrelated sources. Taking the number of cases with clustered genotypes into account, the RTIwas calculated and gave a result of 0.12.

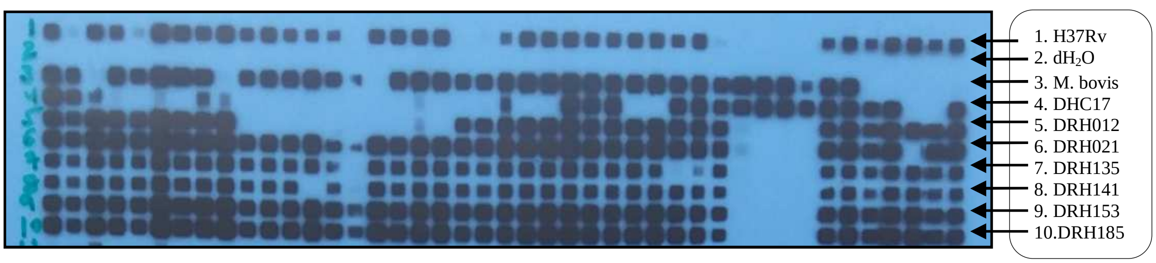


Figure 7. Part of spoligotyping autorad for some of the M. tb isolates (4-10) at ALIPB, AAU

Lane 1 and 3 are positive controls while lane 2 is a negative control. Presence of spacer on the autorad is indicated as a black square while its absence is represented by white space

The study showed that there was a statistically significant difference in the proportion of clustering across Oromia Special Zone and South Wollo Zone isolates (p-value = 0.000). All the isolates from Oromia Special Zone were Orphans where as 14.3% of the isolates from South Wollo Zone had shared international type which showed that there was a statistically significant difference (p-value = 0.000) among the orphan and those that have shared international type across the two study sites (Table 7).

Table 7. Proportion of clustering and shared international type among Oromia Special Zone and South Wollo Zone in Northeastern Ethiopia

	Variables	Oromia Special Zone	South Wollo Zone	Total	
		Number (%)	Number (%)		P-value
Clustering	Clustered	5 (5.1%)	14 (14.3%)	19 (19.4%)	0.000
	Singleton	23 (23.5%)	56 (57.1%)	79 (80.6%)	
	Total	28 (28.6%)	70 (71.4)	98 (100%)	
SIT	Absent/Orphan	28 (28.6%)	56 (57.1%)	84 (85.7%)	0.000
	Shared	0 (0/0%)	14 (14.3%)	14 (14.3%)	
	Total	28 (28.6%)	70 (71.4%)	98(100%)	

Identification of the strains using web-based SPOTCLUST database indicated more than 73% (76/104) of them belonging to the family T1, family33, H37Rv and CAS families accounted for 24.0% (25/104), 23.1% (24/104), 14.4% (15/104) and 11.5% (12/104), respectively. The remaining less proportion belongs to the families T3 6.7 %(7/104), Haarlem3 5.8% (6/104), EAI4 and Haarlem1 4.8% (5/104) each, and others (EAI5, family34, x1, LAM8 and LAM9) with 1.0% (1/104), each. The major lineages identified by the study were: Lineage 1 or Indo-Oceanic (IO), Lineage 3 or East-African India (EAI) and Lineage 4 or Euro-American (EUA). Of these, the most predominant lineages by a conformal Bayesian network (CBN) was Lineage 4 (Euro-American) with a proportion of 65.4% (68/104) followed by ancestral Lineage 1 (Indo-Oceanic), 20.2% (21/104). The modern lineage 3 (East-African Indian) was also identified by the finding with less proportion. No Lineage 2 (East-Asian) and Lineage 7 (Ethiopian type) were identified by this study. Further characterization to sub-lineage level by knowledge-based Bayesian network (KBBN) showed a leading T sublineage from EUA genotype with 23.1% (24/104) followed by CAS1-Delhi 11.5% (12/104) from EAI. The T3-ETH sublineage was also the second dominant proportion among the EUA Lineages (Table 8).

Table 8. The major lineages, sublineages and its proportion

Major lineages (CBN)	Sublineages (KBBN)	Number of strains (Percentage)
Lineage 1 (Indo-Oceanic)	EAI1-SOM	1(1.0)
	Manu_ancestor	3(2.9)
	Manu1	5(4.8)
	Manu2	9(8.7)
	Manu3	11(10.6)
Sub-total		**29 (27.9)**
Lineage 3 (East-African Indian)	CAS	3(2.9)
	CAS1-Delhi	12(11.5)
	CAS1-Kili	5(4.8)
Sub-total		**20(19.2)**
Lineage 4 (Euro-American)	H	3(2.9)
	H1	4(3.8)
	H3	3(2.9)
	H37Rv	3(2.9)
	H3-Ural-1	1(1.0)
	H4-Ural-2	3(2.9)
	LAM7_TUR (Turkey)	1(1.0)
	T	24(23.1)
	T1-RUS2	1(1.0)
	T3	1(1.0)
	T3-ETH	7(6.7)
	T4	3(2.9)
	T-Tuscany	1(1.0)
	Turkey	1(1.0)
Sub-total		**55(52.9)**
Grand total		**104 (100)**

Key: CAS=Central Asian; T=Tuscany, EAI=East-African Indian, LAM=Latin American, H=Haarlem

Using the fourth international spoligotyping database (SpolDB4), there were 14 already recorded isolates as a distinct spoligotype patterns shared international types (SIT) namely SIT 149 (3 isolates), SIT 53 and 1378 (2 isolates each), SIT 1802, 47, 612, 1166, 1251, 1475 and

1547 (one isolate each). Majority of the isolates 86.5% (90/104) were found as orphan, previously unreported isolates (Appendix VII).

Table 9. Description of some spoligotype patterns, lineages and shared-types among 104 valid M. tuberculosis isolates collected from northeast Ethiopia, April 2015 to January 2017

Webdings format	Octal code	Family	Major Lineage	Sub-lineage	SIT	No*
□□■■□■■□■□■■■□□■□□□□□□■■■■■■■■■□□□□□■■■■□□□	155344037740740	H37Rv	EUA	T-Tuscany	Orphan	1
■□□□□□□□■■■■■■□■■□■□■■■■■■□■□□□■□□□□■■■□■■■	401766576420731	Haarlem3	EUA	H4-Ural-2	Orphan	1
■□■□□■□□□□□□□□□□□□□□□□■■■■■■■■□□□□□■■■■■■■	510000017740771	T3	EUA	T1-RUS2	Orphan	1
■□■□□■□□■□□□□□□■□□□□□□□■■■■■□■■□□□□□□□■□■■□■	511004037300261	H37Rv	EUA	H	Orphan	1
■□■□□■□□■□■■■□□■■□□□□□□□□□□□□□□□□□□■■■■■■■	511346000000771	LAM8	EAI	CAS	Orphan	1
■□■□□■□□■■■■■■□■■■■□■■□□□□□□□□□□□□■■□□■■■■■	511767540003171	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■□□■■■■■■□■■■■□■■□□□□□□□□□□□□■■■■■■■■■	511767540003771	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■□■■□■■■■□■■■■□□□□□□□□□□□□□□□■■■■■■■■	513367400001771	CAS	EAI	CAS1-Kili	Orphan	1
■□■□□■□■■□■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■■■	513377400001771	CAS	EAI	CAS1-Kili	Orphan	2
■□■□□■□■■■■■■■□■■■■□■■□□□□□□□□□□□□■■■□■■■■■	513767540003571	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■■□■□□□□□□■□□□□□□□■■■■■□■■□□□□□□□■□■■□■	515004037300261	H37Rv	EUA	H	Orphan	1
■□■□□■■□■□■□□□□■■■□□□□■■■■■■■■■□□□□□□□■□■■□■	515207037740261	H37Rv	EUA	T	Orphan	2
■□■□□■■□■□■□□□□■■■□■□□■■■■■■■■■□□□□□□□■□■■□■	515207237740261	T1	EUA	T	Orphan	1
■□■□□■■□■□■■■□□■■□■□□□■■■■■■■■■□□□□□□□■■■■□■	515346437740361	H37Rv	EUA	T	Orphan	1
■□■□□■■□■□■■■□□■■■□■■□■■■■■■■■■□□□■□□■□■■□■	515347337742261	Family 33	EUA	T4	Orphan	1
■□■□□■■□■□■■■□□■■■□■■■■■■■■■■■■□□□■□□■□■■□■	515347377742261	Family 33	EUA	T4	Orphan	2
■□■□□■■□■□■■■□□■■■■■■□■■■■■■■■■□□□■□■■□■■□■	515347737742661	Family 33	EUA	Manu2	Orphan	1
■□■□□■■□■□■■■□□■■■■■■■■■■■■■■■■□□□■□□■□■■□■	515347777742261	Family 33	EUA	Manu2	Orphan	2
■□■□□■■□■□■■■□■■■■■■■■■■■■■■■■■■□□■□■■□■■■■	515357777762671	Family 33	EUA	Manu2	Orphan	1
■□■□□■■■■□■■■□□■■■■□□□□□■□□■■■■□□□□□□□■□■■■■	517347404740271	LAM9	EUA	Turkey	Orphan	1
■□■□□■■■■□■■■□□■■■■□□■■■■■■■■■■■□■□■□■■□■■□■	517347477752661	Family33	IO	Manu1	Orphan	1

key: EAI = East-African India; EUA = Euro-American ; IO = Indo-Oceanicand No* = number of isolates with similar pattern.

5.2.3. Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat

Of the 69 MGIT positive isolates 56 had valid amplification products of the 24-loci MIRU-VNTR while the remaining 13 isolates had either incomplete or negative MIRU-VNTR profiles even after repeating the test. The locus band was absent for one of the 24 loci (MIRU-VNTR locus 4052) with the oligos sequence FW(5'-AACGCTCAGCTGTCGGAT-3') and REV(5'-CGGCCGTGCCGGCCAGGTCCTTCCCGAT-3') in the gel for all isolates. The MIRU-24 locus of different *M. tuberculosis* strains was amplified by PCR and separated by agarose gel electrophoresis. Each strain had a different allele number ranged from 0-9 repeats. The two molecular markers used were a 50 bp and 100 bp (Figure 8). Highly diverse genotypes were displayed with all the valid patterns as unique and no clustered isolates were detected. The discriminatory efficiency of 24-loci MIRU-VNTR and a combination of both the MIRU-VNTR and spoligotyping in this study was found as the highest with Hunter-Gaston discriminatory index (HGDI) as 1.000 in each case. It was higher than that of spoligotyping discriminatory power (HGDI = 0.996).

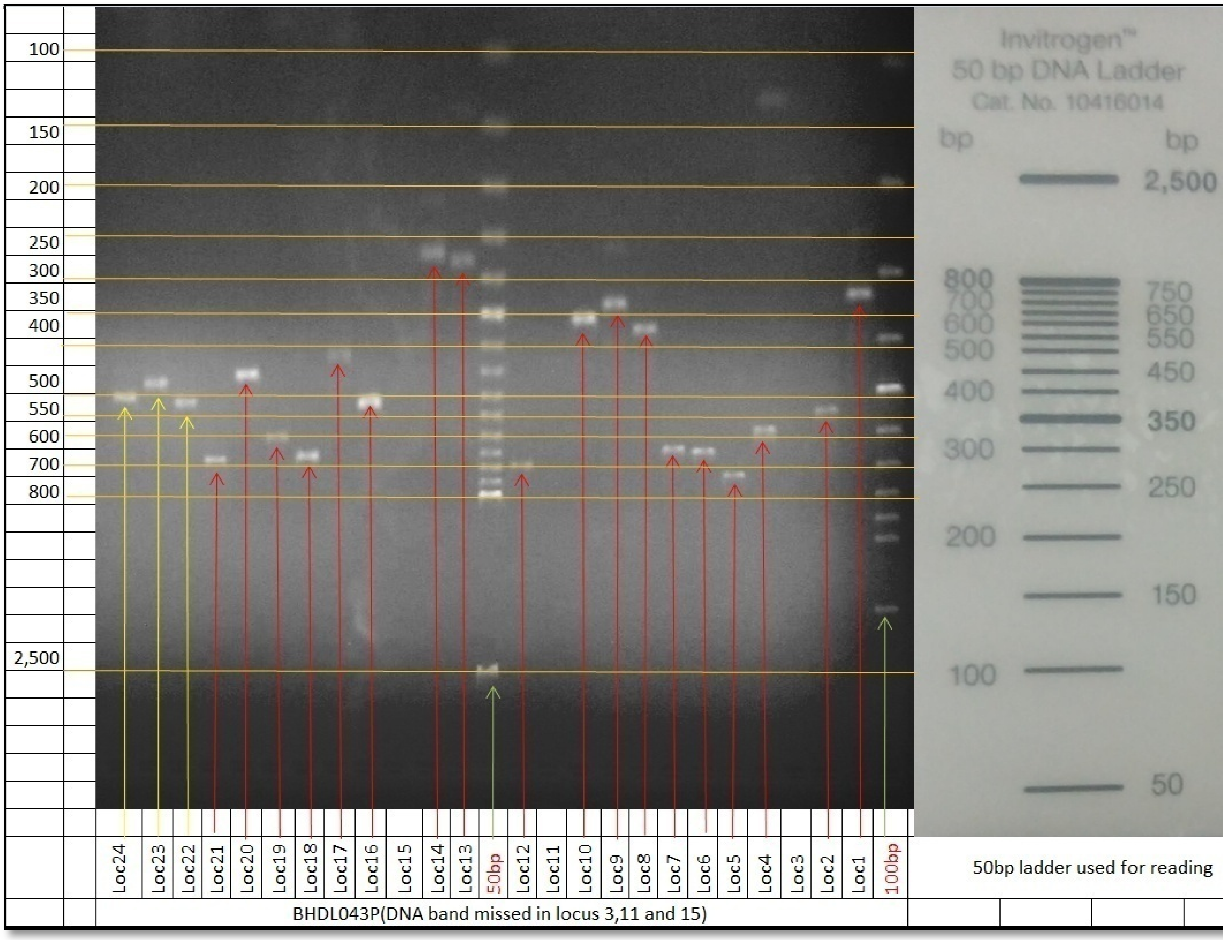
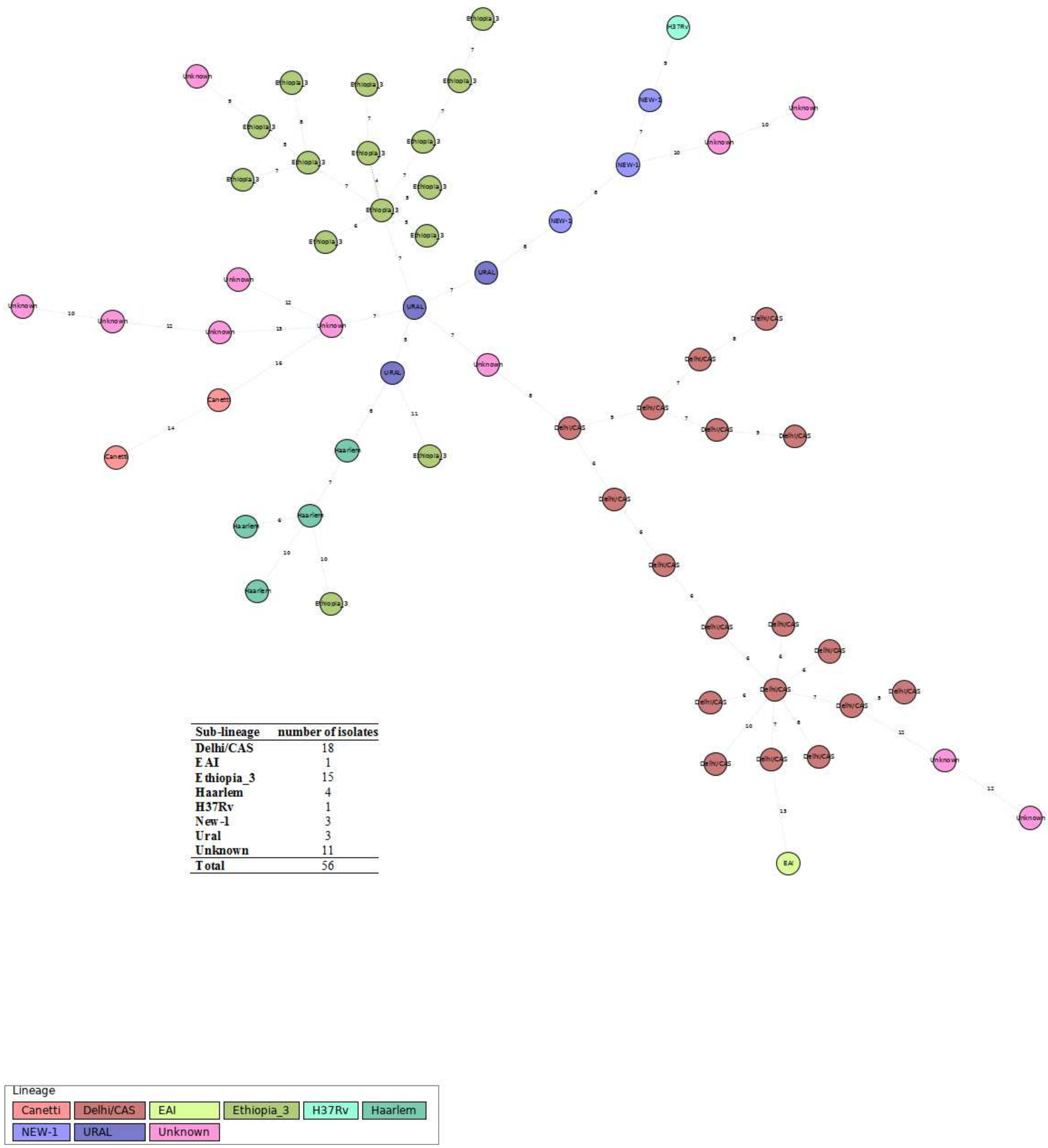


Figure 8. Photographic image of the gel for determination of allelic numbers in 24-loci MIRU-VNTR and the missed bands were repeated

A) Minimum spanning tree (MST) analysis

The MST is used to determine the evolutionary relationship among the strains using MIRU-VNTR*plus* data. None of the strains form a distinct complex as indicated by MST (Figure 9).



Sub-lineage	number of isolates
Delhi/CAS	18
EAI	1
Ethiopia_3	15
Haarlem	4
H37Rv	1
New-1	3
Ural	3
Unknown	11
Total	56

Figure 9. Minimum spanning tree of the 56 MIRU-VNTR valid strains isolated from South Wollo and Oromiya Special Zone, Northeastern Ethiopia

Sub-lineages of the strains were colored differently (the colour code is indicated at the bottom) with an additional sub-lineages of *M. canetti* used as a reference standerd. The Canneti was used as an ancestor from the MIRU-VNTR website online. The circle (node of the tree branch) represents each individual pattern and its equal size represents the same number of isolates which is one. Similarly, equal length of the branch represents distance between the patterns.

Based on the 56 isolates under study for spoligotying and MIRU-VNTR analysis one of the isolates was not valid for the spoligotyping patterns. The 24-loci MIRU-VNTR genotyping showed all the isolates as unique and no recent transmission among the TB cases. The SITVIT analysis for the remaining isolates identified a diverse sub-lineages as T (20%), CAS1-Delhi (16.4%), Manu3 (12.8%), CAS1-kili (9.1%), Manu2 (7.3%), CAS (5.5%), H1(5.5%), T3-ETH (5.5%), H3 (3.6%), H(3.6%) and the remaining small proportion Manu3, EAI1-SOM, , H3-Ural-1, Manu-ancestor, T-Tuscany and and Turkey were 1.8% each. Based on 24-loci MIRU-VNTR*plus* analysis, the identified lineages were Delhi/CAS (32.1%), Ethiopia_3 (26.8%), Unknown (19.6%), Haarlem and NEW-1 4(7.1% each), Ural (5.4%) and EAI (1.8%) (Figure 10). The result of MIRU-VNTR showed that isolates with similar spoligotype pattern (clusterd isolates) had unique copies (singletons) under the 24-loci MIRU-VNTR analysis in the study. All isolates in the study were new types to MLVA MtbC15 and 30 isolates were new types to MLVA MtbC9. These new types were submitted and confirmed to the MIRU-VNTR*plus* nomenclature data base.

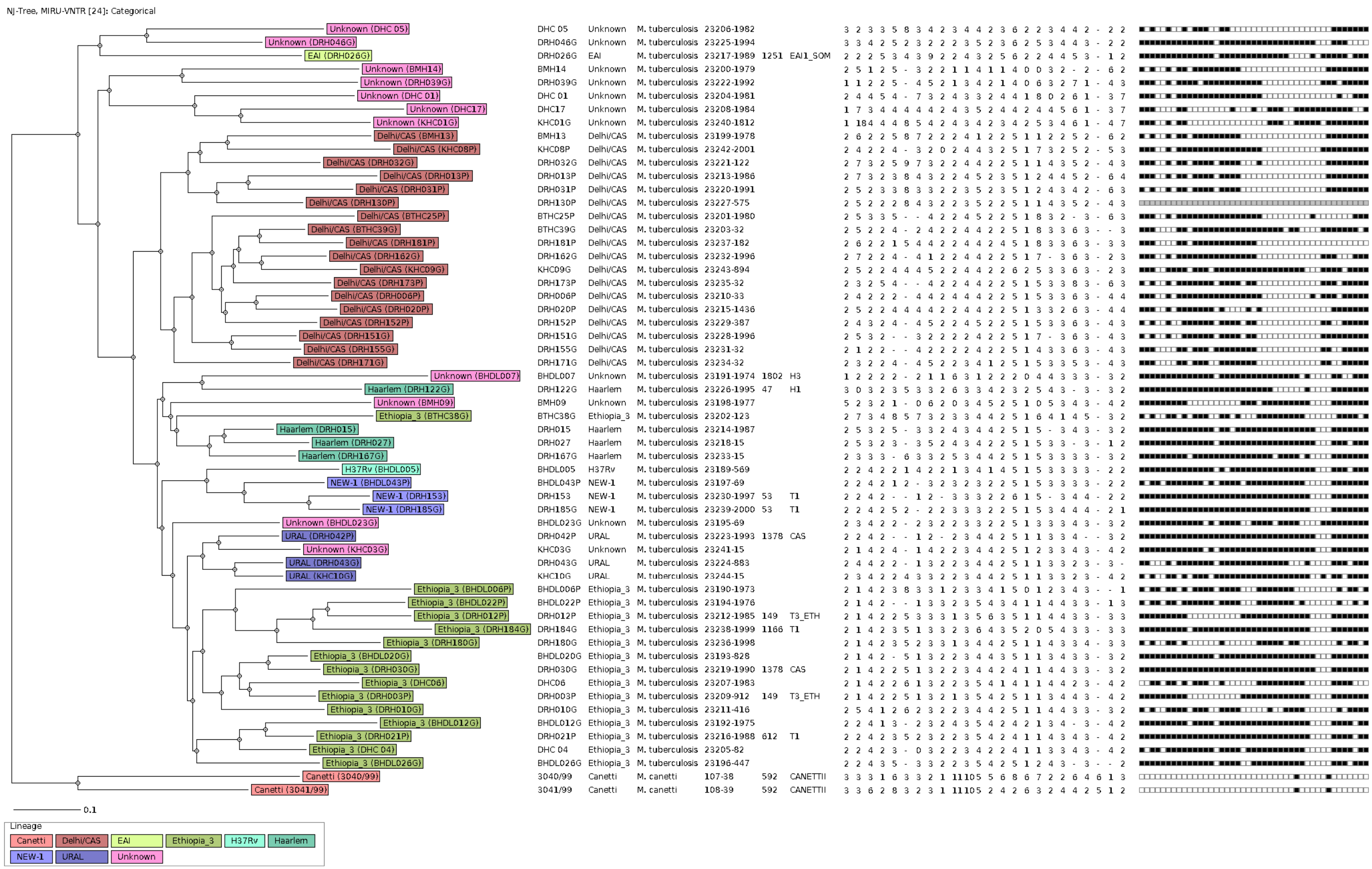


Figure 10. Neighbor-joining tree based on MIRU-VNTR 24-loci pattern of 56 isolates and two M. canetti to reroot the tree as reference from Northeast Ethiopia

5.3. Drug Sensitivity Profiles of *M. tuberculosis* Isolates

Among the 384 TB samples collected, 95 were identified by rapid molecular diagnostic GeneXpert at DRH while the rest were detected by smear microscopic test. The overall LJ-culture positive sample was 29.2% (112/384). Of the Xpert MTB/RIF tested samples, two were identified as RIF resistant. Twenty two of the 95 GeneXpert samples were LJ-culture positive and used for further drug sensitivity test using the GenoType MTBDR*plus* assay and the conventional BACTEC MGIT 960 System. Both methods confirmed the two RIF resistant samples by Xpert as drug resistant and in addition they also detect one sample as multidrug resistant.

The sub-culture test by MGIT gave 61.6% (69/112) as positive and 64 of these sub-culture positive samples as from new TB cases and the rest five as from retreatment patients. Sixty five samples were correctly identified as *M. tb* and30 of the MGIT sub-culture negative samples were detected as *M. tb* positive by the GenoType MTBDR*plus* assay. The remaining 13 of the 112 LJ-culture positive samples were negative to MGIT sub-culture and also not detected as *M. tb* by LPA. Of the 112 isolates tested by line probe assay 15.2% (17/112) have no TUB band. These translate to sensitivity, specificity, positive predictive value and negative predictive value of 94.2%, 30.2%, 68.4% and 76.5%, respectively. There was a fair agreement (Kappa = 0.276; $P < 0.001$) between the two methods (MGIT and LPA) in detecting the *Mycobacterium* when MGIT was used as the reference standard in the analysis (Table 10).

Table 10. M. tuberculosis detection rate by LPA and MGIT using the 112 LJ-culture positive samples

LPA detection	MGIT detection		Total (=112)
	Detected (=69)	Not detected (=43)	
Detected	65	30	95 (84.8%)
Not detected	4	13	17 (15.2%)

Key: LPA=Line probe assay; MGIT=*Mycobacterium* growth indicator tube

Sensitivity test done by LPA detected 16.8% (16/95) as drug resistant of which 18.8% (3/16) were from retreatment cases. The four first line anti-TB drugs (STM, INH, RIF and ETM) by conventional BACTEC MGIT 960 also identified comparable proportion 15.9% (11/69) as drug resistant with 8 and 3 samples as from the new and retreatment ones, respectively. More than half of the sub-culture positive samples by MIGIT 56.5% (39/69) were from Dessie referral hospital and all isolates from BMH were detected as drug resistant by either MGIT, LPA or both (Table 11).

Table 11. Drug susceptibility pattern using genotypic line probe assay and phenotypic Mycobacterium growth indicator tube in relation to the health institutes of Northeastern Ethiopia

Health Institutes	**LPA**		**MGIT**	
	Sensitive No.(%)	Resistant No.(%)	Sensitive No.(%)	Resistant No.(%)
BHDL	10 (10.6)	2 (2.1)	10 (14.3)	2 (2.9)
BMH	-	4 (4.3)	-	2 (4.3)
BTHC	8 (8.5)	2 (2.1)	5 (8.6)	1 (1.4)
CRHC	-	1 (1.1)	-	-
DHC	7 (7.4)	1 (1.1)	5 (7.1)	-
DRH	48 (51.1)	6 (6.4)	33 (48.6)	6 (7.1)
JHC	-	-	-	-
KHC	6 (6.4)	-	5 (7.1)	-
Total No.(%)	79 (83.2)	16 (16.8)	58 (84.1)	11 (15.9)

Key: BHDL= Biqat higher diagnostic lab; BMH= Boru Meda hospital; BTHC= Bati Town health centre; CRHC= Chefa Robit health center; DHC= Dessie health center; DRH= Dessie referral hospital; JHC: Jaraniyo health center; KHC= Kemise health center; LPA=Line probe assay; MGIT=*Mycobacterium* growth indicator tube

Drug resistance was detected in 8 common isolates of MGIT and LPA test. In addition, the LPA identified 2 isolates as susceptible which were resistant by MGIT and also no TUB band was observed in LPA strip for one isolate. Six of the resistant isolates detected by LPA were not tested for their sensitivity by MGIT due to their negative sub-culture result and 2 of the isolates resistant by LPA were susceptible to MGIT (Table 12).

Table 12. Drug resistant isolates detected by phenotypic MGIT and genotypic LPA techniques

No.	Drug resistant sample code	Sensitivity test	
		MGIT	LPA
1	BHDL007	Resistant(S)	no TUB band
2	BHDL020	Resistant(SIRE)	Resistant(R)
3	BHDL030	Susceptible	Resistant(R)
4	BMH08	sub-culture neg.	Resistant(IR)
5	BMH09	Resistant(SIRE)	Resistant(IR)
6	BMH13	Resistant(SIRE)	Resistant(IR)
7	BMH23	sub-culture neg.	Resistant((IR)
8	BTHC01	Resistant(R)	Resistant(R)
9	BTHC09	sub-culture neg.	Resistant(R)
10	CRHC02	sub-culture neg.	Resistant(IR)
11	DHC21	sub-culture neg.	Resistant(IR)
12	DRH021	Resistant(E)	Susceptible
13	DRH030	Resistant(IE)	Susceptible
14	DRH032	Resistant(SIRE)	Resistant(IR)
15	DRH038	Susceptible	Resistant(I)
16	DRH046	Resistant(SIR)	Resistant(R)
17	DRH122	Resistant(I)	Resistant(I)
18	DRH123	sub-culture neg.	Resistant(R)
19	DRH161	Resistant(IRE)	Resistant(IR)

Key: BHDL= Biqat higher diagnostic lab; BMH= Boru Meda hospital; BTHC= Bati Town health centre; CRHC= Chefa Robit health center; DHC= Dessie health center; DRH= Dessie referral hospital; LPA=Line probe assay; MGIT=*Mycobacterium* growth indicator tube

Greater proportion (8.4%) of the LPA tested isolates were multidrug resistance than mono-resistant. Of these, 5.3% (5/95) was among new patients and 3.2% (3/95) of the MDR as among retreatments. Rifampicin and isoniazid mono-resistance accounted for 6.3% (6/95) and 2.1% (2/95), respectively. The LPA test detects 57.1% (8/14) of rifampicin resistant isolates had multidrug resistant TB. On the other hand, all of the sub-culture positive samples by BACTEC MGIT 960 underwent 276 tests and 15.9% (11/69) of the samples were detected as resistant to any of the four anti-TB drugs. More resistance was detected 5.8% (4/69) to the four first-line antimicrobial drugs (INH + RIF + STM + EMB) by the MGIT test than their monoresistance (INH =1.4%, RIF =1.4%, STM=1.4%, EMB=1.4%) (Figure 11). The frequency of resistance to isoniazidniazd, rifampicin, streptomycin and ethambutol was in a comparable proportion 11.6% (8/69), 10.1% (7/69), 10.1% (7/69) and 8.7% (6/69); respectively. Greater proportion of resistance was detected in females 22.6% (7/31) than males 14.1% (9/64) by LPA test and also comparable proportion of drug resistance was detected in females 20% (4/20) than males 14.3% (7/49) by MGIT.

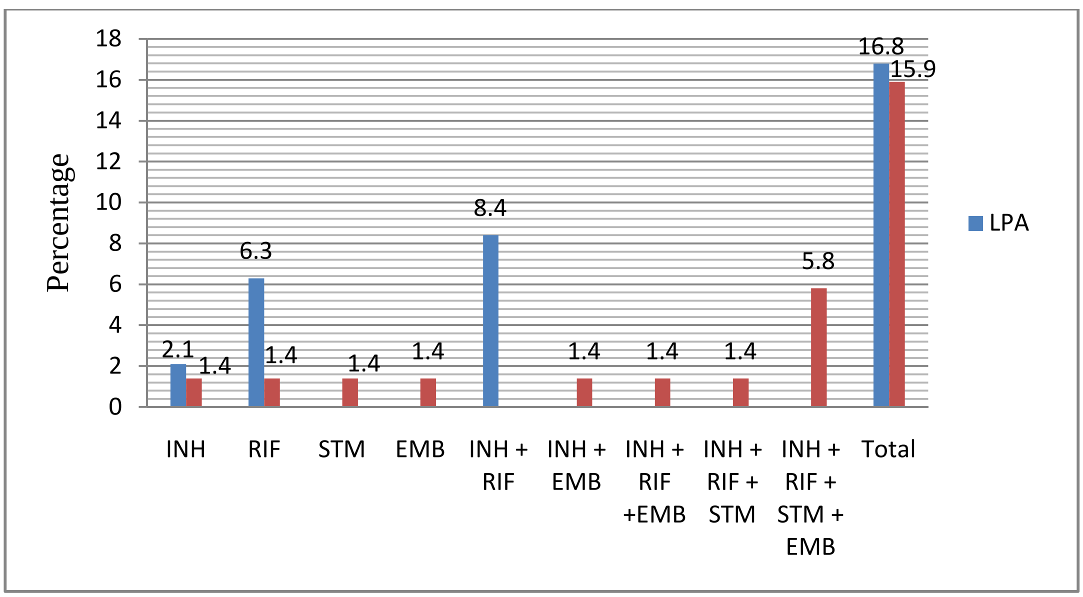


Figure 11. Drug resistance pattern by genotypic line probe assay (LPA) and phenotypic Mycobacterium growth indicator tube (MGIT) from Northeastern Ethiopia, April 2015 to January 2017

Key: INH= Isoniazidniazd; RIF=Rifampicin; STM= Streptomycin EMB= Ethambutol; LPA= Line probe assay; MGIT=*Mycobacterium* growth indicator tube

As compared to BACTEC MGIT 960, the sensitivity and specificity of the GenoType MTBDR*plus* assay for the detection of RIF-resistant *M. tb* isolates was 100 and 98.3%, respectively. Similarly, the sensitivity and specificity for detection of INH-resistance was 75 and 98.2%, and for MDR resistance 83.3 and 100%, respectively. Kappa value showed that there is almost a perfect agreement between the two methods in detecting RIF and MDR where as there is a substantial agreement for the detection of isoniazid (Table 13).

Table 13. Performance of GenoTypic MTBDRplus assay for detection of RIF, INH and MDR resistance in comparison to phenotypic BACTEC MGIT 960 System

Drugs	**MTBDR*plus***	**BACTEC MGIT 960**		**Sensitivity(%)**	**Specificity(%)**	**PPV(%)**	**NPV(%)**	**Kappa**
		Resistant	Sensitive					
RIF	Resistant	7	1	100	98.3	87.5	100	0.925
	Sensitive	0	57					(p<0.001)
INH	Resistant	6	1	75	98.2	85.7	96.6	0.774
	Sensitive	2	56					(p<0.001)
MDR	Resistant	5	0	83.3	100	100	98.3	0.901
	Sensitive	1	59					(p<0.001)

In comparison to MGIT, the drug sensitivity of LPA at *katG* gene was found as 75% detection and that of *inhA* promoter region was 12.5%. The sensitivity of LPA to both *katG* and *inhA* was also 12.5%. This finding also revealed that the *katG*, *inhA*, both *katG* and *inhA* results of LPA test had almost perfect agreement, of susceptibility to the MGIT test (Table 14).

Table 14. Detection of isoniazid (katG, inhA and both katG and inhA) resistance by LPA with its resistance by MGIT testing method

LPA		MGIT isoniazid DST		Sensitivity	Specificity	Predictive value		Kappa
		Resistant = 8	Sensitive = 57			Positive	Negative	
katG	Resistant	6	0	75	100	100	96.6	0.840 (P<0.001)
	Sensitive	2	57					
inhA	Resistant	1	1	12.5	98.2	50	88.9	0.159 (P=0.099)
	Sensitive	7	56					
KatG and *inhA*	Resistant	1	0	12.5	100	100	89.1	0.2 (P=0.007)
	Sensitive	7	57					

5.3.1. Resistance and mutation patterns of rifampicin and isoniazid using the GenoType MTBDR*plus* assay

For the line probe assay, single probe resistance was detected in 25.0% (4/16) of the isolates and two or more probe resistance was detected in the remaining drug resistant isolates. In addition, single probe mutation was detected in 12.5% (2/16) of the drug resistant isolates. In 10 of the RIF resistant isolates, band 8 (WT8) was omitted with an additional miss of WT3, WT4 and WT6 probe in the *rpoB* gene of three isolates. There were also an omission of WT3 and WT7 probe for 3 RIF resistant isolates and no gain of mutant probe was detected for all the 13 RIF resistant isolates in the *rpoB* gene region. Concerning isonizid resistance, the resistance was further identified using *katG* and *inhA* gene region. Of the 12 INH-resistant isolates, *katG* resistance was detected in 66.7% (8/12) of the isolates by omitting WT probe at codon 315 and mutations were detected in two of the isolates at Codon S315T1. Mutation was identified in five isolates of the *inhA* gene region at WT1 (-15/-16) and an additional mutation to one isolate at WT2 (-8). Addition of specific *inhA* mutations were not observed in place of the omitted probes at the *inhA* promoter region of the gene (Table 15).

Table 15. Result of GenoType MTBDRplus assay in RIF and INH resistant isolates

Genes	Band (gene region/mutation)	**LPA drug resistant detected isolates**															
		BHDL		BMH				BTHC		CRHC	DHC	DRH					
		20	30	8	9	13	23	1	9	2	21	32	38	46	122	123	161
rpoB		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT1 (506-509)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT2 (510 - 513)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT3 (513 -517)	S	S	S	S	**R**	S	S	S	**R**	**R**	S	S	S	S	S	**R**
	WT4 (516 - 519)	S	S	S	S	**R**	S	S	S	S	**R**	S	S	S	S	S	S
	WT5 (518 - 522)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT6 (521 -525)	S	S	S	S	S	S	S	**R**	S	S	S	S	S	S	S	S
	WT7 (526 - 529)	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	S
	WT8 (530 -533)	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	S	S	S	S	S
	MUT1 (D516V)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT2A (H526Y)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT2B (H526D)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT3 (S531L)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
katG		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT (315)	**R**	S	S	R	**R**	**R**	S	S	S	S	**R**	S	S	**R**	**R**	**R**
	MUT1 (S315T1)	S	S	S	S	S	**R**	S	S	S	S	S	S	S	S	**R**	S
	MUT2 (S315T2)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
inhA		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	WT1 (-15/-16)	S	S	**R**	S	**R**	S	S	S	**R**	**R**	S	**R**	S	S	S	S
	WT2 (-8)	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	S	S
	MUT1 (C15T)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT2 (A16G)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT3A (TBC)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MUT3B (TBA)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Key: S= susceptible; R= resistant; MUT=Mutant; WT=Wild type

5.3.2. Genetic diversity for the drug resistant Mycobacterium tuberculosis

Molecular characterization of the drug resistant *Mycobacterium* was done using spoligotyping and the major lineages were identified as Euro-American (EA), East-African-Indian (EAI) and Indo-Oceanic (IO) by Conformal Bayesian network (CBN). Of these lineages 76.5% (13/17) were Euro-American followed by Indo-Oceanic 17.6% (3/17) lineages. Most of the drug resistant isolates 76.5% (13/17) had no SIT number indicating that they are orphans. Family 33 is also the predominant family identified in this study (Table 16).

Table 16. Spoligotyping result of drug resistant isolates from Northeastern Ethiopia

Resistant Isolates	Family	Major Lineage by CBN	sub-lineage /clade	SIT
BHDL007	*M. tuberculosis* Haarlem3	Euro-American	H3	1802
BHDL020	*M. tuberculosis* T1	Euro-American	Manu3	
BHDL030	Family 33	Euro-American	Manu2	
BMH08	*M. tuberculosis* H37Rv	Euro-American	T	
BMH09	*M. tuberculosis* T3	Euro-American	T3-ETH	
BMH13	*M. tuberculosis* CAS	East-African-Indian	CAS1-Kili	
BMH23	*Family 33*	Euro-American	Manu2	
BTHC09	*M. tuberculosis* T3	Euro-American	T1-RUS2	
CRHC02	*Family33*	Indo-Oceanic	Manu1	
DHC21	Family 33	Euro-American	T4	
DRH021	*M. tuberculosis* T1	Euro-American	T	612
DRH030	Family33	Euro-American	Manu2	1088
DRH032	*M. tuberculosis* EAI4	Indo-Oceanic	CAS1-Kili	
DRH038	*M. tuberculosis* Haarlem3	Euro-American	X1	
DRH046	*M. tuberculosis* T1	Euro-American	Manu3	
DRH122	*M. tuberculosis* Haarlem1	Euro-American	H1	47
DRH123	*M. tuberculosis* Haarlem1	Indo-Oceanic	H1	

The association between any drug resistance and TB type showed a varied proportion but with no statistically significant difference among pulmonary and extra-pulmonary TB cases ($\chi 2$: 0.233; p = 0.629). In addition, there was also no statistically significant association between any anti-TB drug resistant isolates identified and the major lineages ($\chi 2$: 0.557; p = 0.757). In fact, it was found that significant association was observed between the drug resistant isolates and the sub-lineages ($\chi 2$: 34.861; p = 0.040). A statistically significant association ($\chi 2$: 4.779; p = 0.029) was also found between the clustered strains and any anti-TB drug resistant isolates (Table 17).

Table 17. Association between any drug sensitivity patterns and TB type, major lineage, sub-lineage and dominant strains

Variable		Any drug resistance				
		Sensitive (%)	Resistant (%)	Total (%)	χ2 (df)	P- value
TB type						
	Extra-pulmonary	10 (76.9%)	3 (23.1%)	13 (14%)		
	pulmonary	66 (82.5%)	14 (17.5%)	80 (86%)	0.233 (1)	0.629
Major lineage by CBN						
	EA	47 (79.7%)	12 (20.3%)	59 (63.4%)		
	IO	15 (83.3%)	3 (16.7%)	17 (19.4%)	0.757 (2)	0.757
	EAI	14 (87.5%)	2 (12.5%)	16 (17.2%)		
Sub-lineage/Clade						
	AFRI	4 (100%)	0	4 (4.3%)		
	CAS	3 (100%)	0	3 (3.4%)		
	CAS1-Delhi	12 (100%)	0	12 (12.8%)		
	CAS1-Kili	2 (40.0%)	3 (60.0%)	5 (5.3%)		
	EAI1-SOM	1 (100%)	0	1 (1.1%)		
	H	3 (100%)	0	3 (3.4%)		
	H1	0	2 (100%)	2 (2.1%)		
	H3	2 (66.7%)	1 (33.3%)	3 (3.4%)		
	H3-Ural-1	1 (100%)	0	1 (1.1%)		
	H37Rv	1 (100%)	0	1 (1.1%)		
	H4-Ural-2	3 (100%)	0	3 (3.4%)		
	Manu_ancestor	0	1 (100%)	1 (1.1%)	34.861(22)	0.040
	Manu1	3 (75%)	1 (25%)	4 (4.3%)		
	Manu2	6 (75%)	2 (25%)	8 (8.5%)		
	Manu3	7 (70%)	3 (30%)	10 (10.6%)		
	PINI	1 (100%)	0	1 (1.1%)		
	T	17 (94.4%)	1 (5.6%)	18 (19.1%)		
	T-Tuscany	1 (100%)	0	1 (1.1%)		
	T1-RUS2	0	1 (100%)	1 (1.1%)		
	T3	1 (100%)	0	1 (1.1%)		
	T3-ETH	6 (85.7%)	1 (14.3%)	7 (7.4%)		
	T4	2 (66.7%)	1 (33.3%)	3 (3.4%)		
	Turkey	1 (100%)	0	1 (1.1%)		
Dominant strains						
	Orphan	66 (86.8%)	13 (76.5%)	79 (84.9%)		
	SIT1166	1 (1.3%)	0 (0.0%)	1 (1.1%)		
	SIT1251	1 (1.3%)	0 (0.0%)	1 (1.1%)		
	SIT1378	1 (1.3%)	1 (5.9%)	2 (2.2%)		
	SIT1475	1 (1.3%)	0 (0.0%)	1 (1.1%)	16.948(10)	0.076
	SIT149	3 (3.9%)	0 (0.0%)	3 (3.2%)		
	SIT1547	1 (1.3%)	0 (0.0%)	1 (1.1%)		
	SIT1802	0 (0.0%)	1 (5.9%)	1 (1.1%)		
	SIT47	0 (0.0%)	1 (5.9%)	1 (1.1%)		
	SIT53	2 (2.6%)	0 (0.0%)	2 (2.2%)		
	SIT612	0 (0.0%)	1 (5.9%)	1 (1.1%)		
Clustering						
	Yes	10 (62.5%)	6 (37.5%)	16 (17.2	4.779 (1)	0.029
	No	66 (85.7%)	11 (14.3%)	77 (82.8		

5.4. Nutritional Status of the Study Patients

The overall mean BMI of TB cases was calculated as 18.2 kg/m^2 and those proportions of chronic energy deficiency ($BMI \leq 18.5 Kg/m^2$) was found as 50.0% (n=192) (Figure 12). Of this chronic energy deficient TB cases mildly (BMI 17.0-18.5kg/m2), moderately (BMI 16.0kg/m2-16.9kg/m2) and severely (BMI <16.0kg/m2) malnourished patients were 61.5% (n=118), 21.9% (n=42) and 16.7% (n=32), respectively.

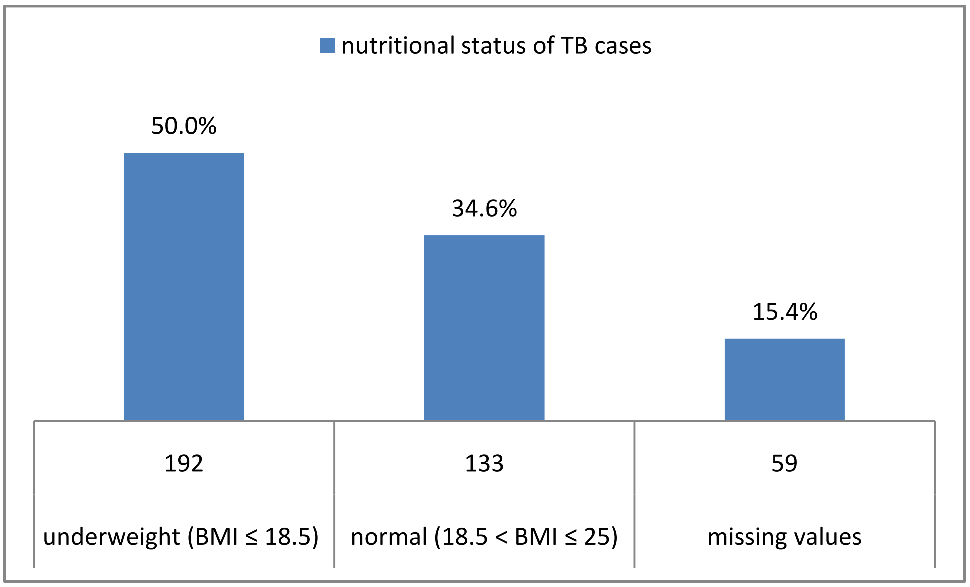


Figure 12. Proportion of malnutrition among tuberculosis cases using body mass index (BMI) parameter, Oromia Special Zone and South Wollo, April 2015 to January 2017

Of 237 TB cases whose faecal specimens were examined, 58.6% (n = 139) were underweight and there was no significant difference (P = 0.821) in the mean body mass index of TB-parasitic co-infected cases (mean = 18.1, SD = 1.3) and TB positive cases without parasitic co-infection (mean = 18.2, SD = 2.1). The BMI of co-infected cases didn't differ significantly from those of non-co-infected cases at different age groups ($\chi 2$ = 4.601, df=3 and P= 0.203) and sex ($\chi 2$ = 0.527, df = 1 and p= 0.468).

Among smear positive TB cases whose MUAC were measured, 72.8% (205/281) of them were undernourished and was not significantly associated with gender ($\chi2$ = 0.831, df = 1 and P = 0.362). When MUAC was used to determine nutritional status, 6.4% of the undernourished TB cases and 2.1% of the normal nourished TB cases had parasitic co infection showing non-significant association ($\chi2$ = 0.056, df = 1 and P = 0.813). As a whole, significant difference was not observed between TB parasitosis co-infections and nutritional status of the TB cases (Table 18).

Table 18. Nutritional status and parasitic co-infection of tuberculosis cases in Oromia Special Zone and South Wollo, Northeastern Ethiopia, April 2015 to January 2017

	Nutritional status using MUAC			**Nutritional Status using BMI**		***P*-value**
	Normal (%)	**Under-nourished (%)**	***P*-value**	**Normal (%)**	**Under-nourished (%)**	
Sex						
Males	38 (24.8)	115 (75.2)	0.362	57 (43.8)	73 (56.2)	0.390
Females	38 (29.7)	90 (70.3)		41 (38.3)	66 (61.7)	
Co-infection status						
Co-infected	6 (25.0)	18 (75.0)	0.813	8 (30.8)	18 (69.2)	0.246
Not co-infected	70 (27.2)	187 (72.8)		90 (42.7)	121 (57.3)	

MUAC: Mid-upper Arm Circumference; BMI: Body Mass Index

Considering our matched criteria set in the methodology part, 86 participants (55 males and 31 females) were used as control groups. The difference between the nutritional status of smear positive TB cases (mean = 21.4, SD = 2.0) and the control groups (mean = 22.9, SD = 2.5) was statistically significant (P = 0. 029) as MUAC parameter was used. However, it is not statistically significant (P = 0. 220) when body mass index is considered with average mean (+ SD) of 18.2 (+ 1.95) and 20.7 (+ 2.73) for TB cases and the control groups, respectively.

5.5. Tuberculosis Parasites Co-infection

A total of 259 faecal samples (142 males and 117 females) were collected and examined. The remaining tuberculosis cases refused to give faecal specimens and some of them were also severely sick and did not provide the specimen. From the study, comparable intestinal parasitic infection was found among pulmonary TB cases 10.5% (19/181) and extra-pulmonary ones 11.5% (9/78) (Table 19).

The overall *M. tb*-parasite co-infection was 10.8% (28/ 259). From the total co-infected cases, 89.3% (25/28) had single parasitic infection, while 7.1% (2/28) had double infection, and 3.6% (1/28) had four infections. The infection of intestinal helminths accounted for 9.7% and those of intestinal protozoa accounted for 1.9%. Although there was greater helminthic co-infection than protozoa co-infection among tuberculosis patients in the study area, the difference was not statistically significant ($\chi 2 = 6.000$, df = 4 and P = 0.199).

Schistosoma mansoni infection was the most prevalent parasitic co-infection (4.25%), followed by *Ascaris lumbricoides* (2.32%). *Trichuris trichiura*, *Enterobius vermicularis* and *Hymenolepis nana* were the other helminthic co-infections whereas *Entamoeba histolytica/dispar* and *Giardia lamblia* cysts were the only detected protozoan parasitic co-infections (Table 19).

Table 19. Parasite species among tuberculosis cases in Oromia Special Zone and South Wollo, Northeastern Ethiopia, April 2015 to January 2017

Types of parasites	Number (percentage) of infection
Schistosoma mansoni	**11 (4.3)**
Ascaris lumbericoides	**6 (2.3)**
Trichuris trichiura	**2 (0.8)**
Enterobius vermicularies	**2 (0.8)**
Giardia lamblia	**2 (0.8)**
Entamoeba histolytica/dispar	**1 (0.4)**
Hookworm	**1 (0.4)**
Schistosoma mansoni and *Giardia lamblia*	**1 (0.4)**
Ascaris lumbericoides and *Enterobius vermicularies*	**1 (0.4)**
Schistosoma mansoni,*Hymenolepis nana, Giardia lamblia* and hookworm	**1 (0.4)**
Total	**28 (10.8)**

The association of TB-parasite co-infection in males (60.7%, 17/28) and females (39.3%, 11/28) was not statistically significant ($\chi2$ = 439, df = 1 and P = 0.507). Similarly, significant difference of *M. tb*-parasitic co-infection was also not observed across different age groups ($\chi2$ = 36.238, df = 40 and P = 0.640).

Mycobacterium Culture positivity, pulmonary and extra-pulmonary tuberculosis

Growth was observed in 29.2% (112/384) samples after culturing all the smear and GeneXpert positive samples on LJ media. Culture positivity was not significantly different as compared to bacterial load under laboratory examination of smear microscopic test (FM; P = 0.455) and GeneXpert (P = 0.427). Thirty six samples had missed grading scale (MGS) of the bacterial load (Figure 13).

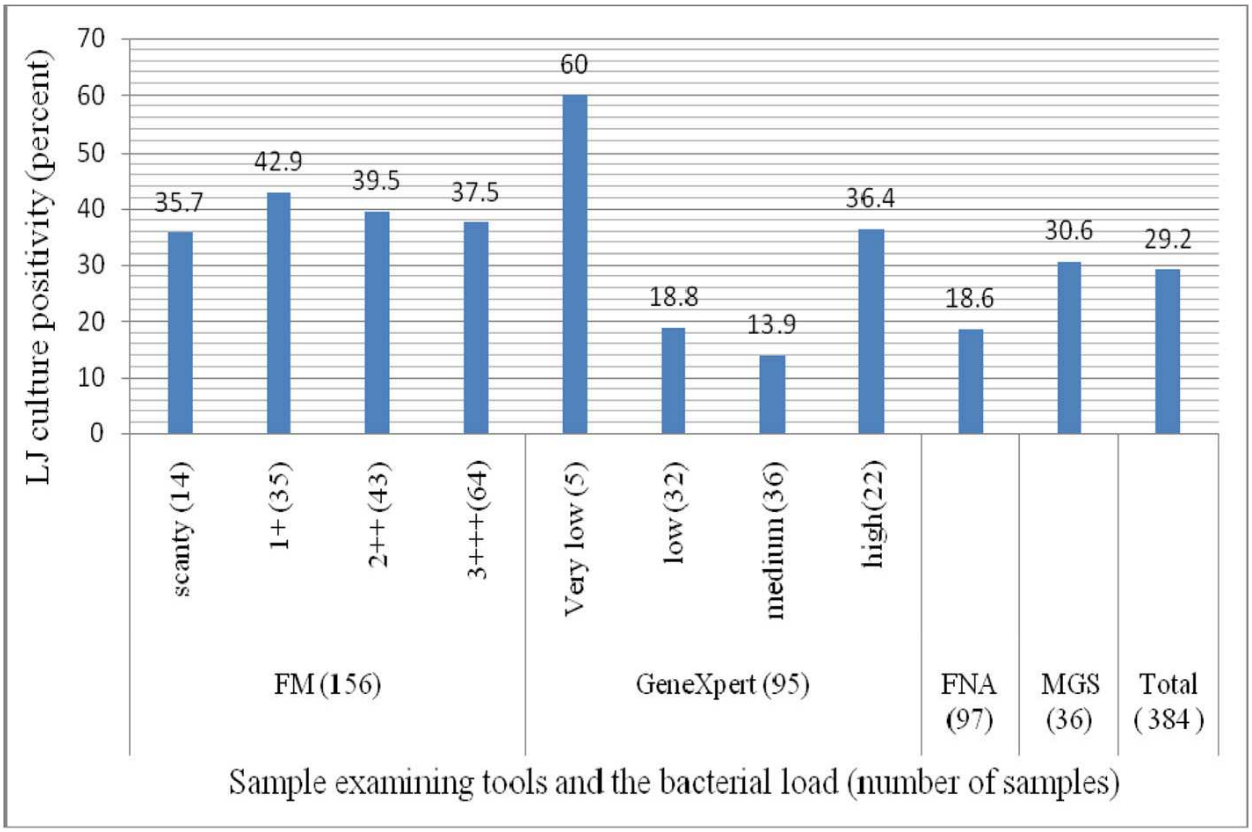


Figure 13. Comparison of results of smear microscopy (FM & FNA) and GeneXpert with bacterial growth on LJ medium, Oromia Special Zone and South Wollo, Northeastern Ethiopia, April 2015 to January 2017

Key: FM=Fluorescent microscope; FNA= Fine-needle aspirates; MGS= Missed grading scale; EPTB= Extra-pulmonary tuberculosis; LNs= Lymphadenitis

Among the EPTB cases, site of infection was detected in 85 participants by the pathologist and TB lymphadenitis (TBLNs) was the most prevalent 85.9% (73/85). Of these TBLNs cervical adenopathy 75.3% (55/73) was the most frequently occurring disease. Of the suspected smear positive EPTB samples, 18.4% were culture positive (Table 20).

Table 20. Site of extra-pulmonary tuberculosis infection and the number of culture positives from BHDL, Oromia Special Zone and South Wollo, Northeastern Ethiopia, April 2015 to January 2017

Site of EPTB infections	Number of infected cases (%)	Number of culture positives (%)
Cervical LNs	55 (56.1)	8 (14.5)
Axilary LNs	8 (8.2)	2 (25.0)
Supraclavicular LNs	6 (6.1)	1 (16.7)
Other LNs*	4 (4.0)	-
Non LN infections	12 (12.2)	4 (33.3)
Not sited (missed)	13 (13.3)	3 (23.1)
Total	98 (100.0)	18 (18.4)

*Inguinal, Sub-mandibular and Anterior neck LNs

Key: BHDL= Biqat Higher Diagnostic Laboratories; EPTB= Extra-pulmonary tuberculosis; LNs= Lymphadenitis

5.6. Assessment of Knowledge, Attitude and Practice of the Patients

Majority of tuberculosis patients had heard about the disease tuberculosis before they were sick and a number of patients also know TB as a contagious but treatable disease. On the contrary, less number (21.3%) of the patients heard about drug resistance bacteria and half of the respondents saw TB patients elsewhere before they were sick. Greater proportion of the respondent patients were new cases than retreatment ones. Interestingly, more than half of the patients had check up for TB at the health institutes as they have prolonged cough (more than 2 weeks) (Table 21).

Table 21. Knowledge and practice related assessment results of TB patients toward tuberculosis

	Questions	Responses		
		Frequency (%)		
No.	**Knowledge based**	**yes**	**no**	**Don't know**
1	Have you ever heard about TB?	243/345 (70.4)	102/345 (29.6)	-
2	Is TB contagious?	260/340 (76.5)	2/340 (0.6)	78/340 (22.9)
3	Is TB a treatable disease?	262/363 (72.2)	3/363 (0.8)	68/363 (18.7)
4	Have you heard drug resistant TB bacteria before?	72/338 (21.3)	266/338 (78.7)	-
5	Is TB a zoonotic disease?	28/314 (8.9)	113/314 (36.0)	173/314 (55.1)
No	**Practice based**	**yes**	**no**	**Sometimes**
1	Have you seen TB patients before?	171/337 (50.7)	166/337 (49.3)	-
2	Have you been sick of TB before this?	34/320 (10.6)	286/320 (89.4)	-
3	Do you check for TB at health institution when you have prolonged cough (more than 2 weeks)?	207/339 (61.1)	39/339 (11.5)	93/339 (27.4)

Majority of the patients 60.4% (204/338) responded that drug resistant bacteria are transmitted from any form of TB patients where as only 2.4% (8/338) responded it comes from those patients who had drug resistance TB. The remaining 28.7% (97/338), 1.5% (5/338) and 0.6% (2/338) of the respondents replied drug resistance is caused by incorrect use of the antimicrobial drugs, non-adherence of the drugs to the prescribed regimen/natural phenomenon due to mutation and do not know how drug resistance is developed, respectively. About 6.5% (22/338) of the patients responded that drug resistance is caused by any of the above two or more alternatives.

More than half 122/213 (57.3%) of the patients know TB is caused by bacteria but greater proportion of them didn't identify the body organs infected by the *Mycobacterium*. Although a large proportion 274/342 (80.1%) were aware that using specific drugs prescribed by health physicians can cure the patients, a number of patients do not know the length of time it took for the treatment of drug resistant and non-resistant bacteria (Table 22).

Table 22. Knowledge and practice of TB patients in Northeastern Ethiopia, April 2015 to January 2017

Variable	Responses	
	Frequency	Percentage
1. Causative agent of TB (n=213)		
a) Bacteria/germ	122	57.3
b) Cold air	20	9.4
c) Shortage of food	3	1.4
d) Smoking	16	7.5
e) Chewing 'Khat'	2	1.0
f) Dust	49	23.0
h) Don't know	1	0.5
2. TB bacteria attacks (n=328)		
a) Lung only	96	29.3
b) Other body part but not lung	20	6.1
c) Both lung and other body part	95	29.0
d) Don't know	116	35.4
e) Others (please specify)	1	0.3
3. How can someone with TB be cured? (n= 342)		
a) By using herbal remedies (traditional medicine)	5	1.5
b) Taking home rest without medicine	10	2.9
c) By praying	2	0.6
d) By using specific drugs given by health centre based on their prescription	274	80.1
e) Do not know	1	0.3
f) Other (please specify):	50	14.6
4. Drug resistant bacteria take (n = 340)		
a) Equal duration as non resistant ones for treatment	6	1.8
b) Less duration than non resistant ones for treatment	5	1.5
c) Greater duration than non resistant ones for treatment	58	17.1
d) Can't be treated	2	0.6
e) Don't know	269	79.1
5. Where do you go first when you feel sick? (n = 340)		
a) Traditional healers'	17	5.0
b) Religious beliefs	11	3.2
c) Private clinics	11	3.2
d) Governmental health institutions like health posts, clinics, health center or hospital	289	85.0
e) Clinics run by non-governmental organization	10	2.9
f) Others	2	0.6

Majority of the respondents 72% (252/350) replied that tuberculosis is non-selective and anybody can be infected by the disease. Less proportion of the respondents gave their views that TB infects poor, homeless, alcoholics, drug users, individuals living with HIV, 'Khat' users and others (Figure 14).

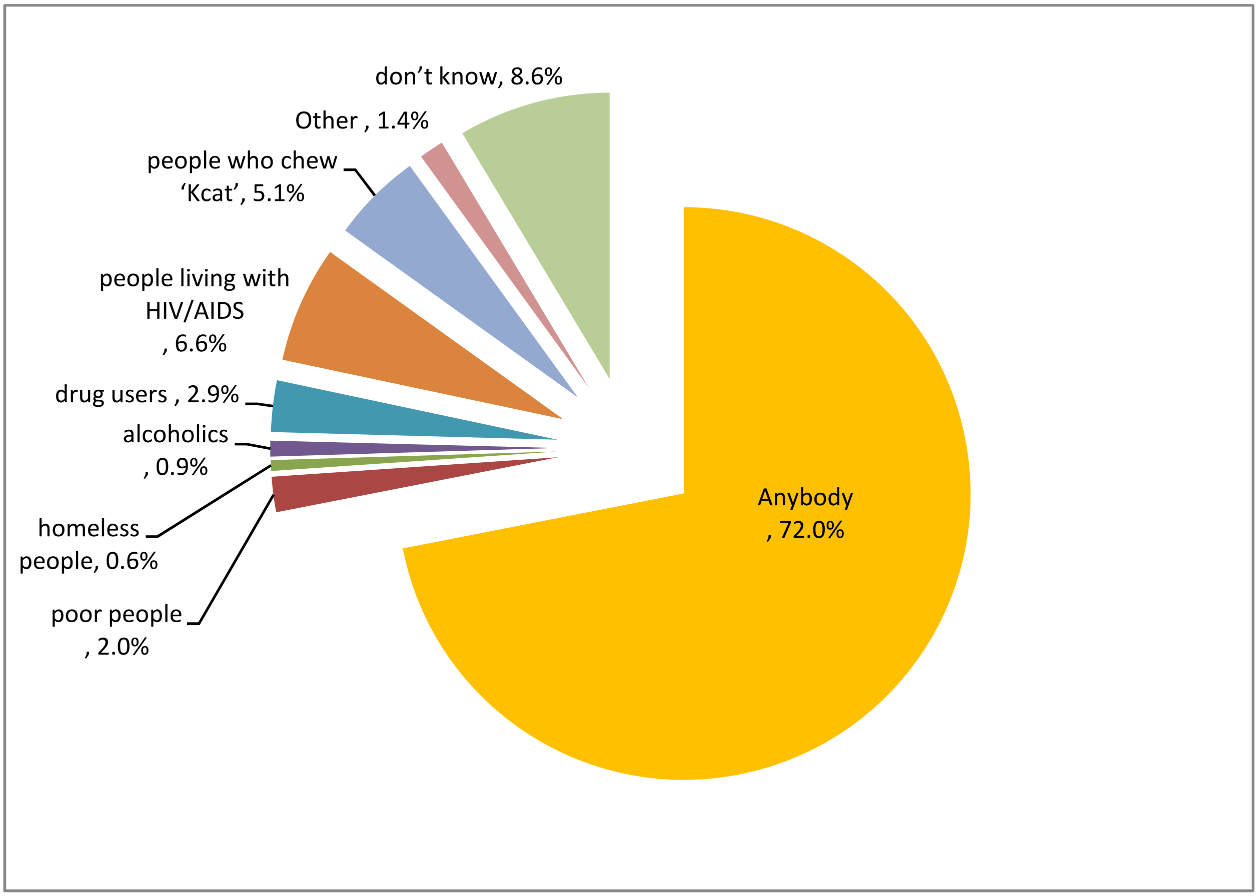


Figure 14. Tuberculosis patients' reflection on TB infection in Northeast Ethiopia, April 2015 to January 2017

6. Discussion

6.1. Demographic Characteristics of the Study Patients

The greater number of TB cases in Dessie Town than in other districts might be due to higher population density in the Town. This is in agreement with WHO report that states the prevalence of TB cases is considerably higher in urban areas than in rural areas (WHO, 2015). In addition, access and better diagnosis with more proximity to the health institutes could be another factor to find higher number of cases in Dessie Town. Majority of the patients were males 55.5% (213/384) than females with comparable proportion to 56.2% (63/112) by Tadesse *et al*., (2016). The male to female ratio of this finding is also the same to bacteriologically confirmed pulmonary TB patients of WHO report for Ethiopia with M:F ratio as 1.2 (WHO, 2019). This greater proportion of male patients to females might be due to biological, social and economic engagement to contact with many people including taking patients into health institutes for treatment. The disease was also more common within age range of 18 - 37 years which could be due to their active movement from place to place for economic purposes that leads them to greater risk of exposure.

6.2. Molecular Epidemiology of Tuberculosis

The detection of *M. tb* from culture positive samples in this study using RD9 was lower than the findings from many other studies. A study from Western (Disassa *et al*., 2015), Northeastern (Birhanu *et al*., 2014), central (Bedewi *et al*., 2017) and Northwestern (Alelign *et al*.,2019) Ethiopia reported a proportion of 97.1% in the first study and 100% in all the rest. On the contrary, there was also a report from Addis Ababa (Negesse *et al*., 2019) that showed RD9 detection typing for *M. tb* with less proportion 47.7% (41/86) than this finding.

The greater variation of spoligotype clustering in this study and many other reports might be due to the difference in geographical study settings. There were higher clustering rate report from many of the studies in Ethiopia with a proportion of 79.3% (223/281); 63.8% (102/160), and 84.7% (50/59) as reported by Bedewi *et al*., 2017; Mekonnen *et al*., 2018 and Molina-Moya *et al*., 2018, respectively. Similarly, most studies outside of the country also reported greater proportion of clusters as by Kisa *et al*., 2012;Ahmed *et al*., 2014; Ali *et al*., 2014 and Carvalho *et al*., 2016 with a proportion of 79% (75/95), 82.2% (111/134), 68.2% (184/270) and 89.2% (33/37) in that order than the overall clustering rate 20.2% (21/104) of this study which is close to 18.8% (6/32) (Birhanu *et al*., 2014) and 23% (6/26) (Chemeda *et al*., 2018) report from Dessie and Addis Ababa, respectively. The findings of all isolates in Oromia Special Zone as Orphan imply that they were not registered in the data base as there was no more study from the area so far. The less clustering rate in Oromia Special Zone was similar to the overall study in this finding. Such less proportion could imply that the *Mycobacterium* infections were from unrelated sources or it might be caused due to latent tuberculosis activation. The predominance of families T1, family33, H37Rv and CAS in line with a systematic review reported by Tulu and Ameni (2018) and a research report by Mihret *et al*. (2012).

The higher proportion of modern strains Lineage 3 (East-African Indian) and Lineage 4 (Euro-American) of the *Mycobacterium* than the ancient Lineage 1(Indio Oceanic) was in agreement with a study from Dessie 71.4% (20/28) (Birhanu *et al*., 2014). This greater proportion of the modern strains to the ancient strains could be due to the recent expansion (Later than 20th Century) of tuberculosis in Ethiopia than the ancient Indio Oceanic ones which is more common in population living around the Indian Ocean (Comas *et al*., 2015). A study from Southwest Ethiopia (Tadesse *et al*., 2017) reported the existence of Lineage 2 (East-Asian) at a low proportion 0.3% (1/333) which was totally absent in the present study implying its lower transmission rate. Absence of Lineage 7 (Ethiopian type) which was commonly reported from the northeastern Ethiopia (Firdessa *et al*., 2013; Mengistu *et al*., 2015; Yimer *et al*., 2015; Yimer *et al*., 2016) might also be due to its less transmission dynamics in the area. Similar molecular study in Dessie was also in agreement with this finding by Birhanu *et al*. (2014) and rare Ethiopian type was reported by Maru *et al*. (2015).

The higher proportion of T sublineage followed by CAS1-Delhi in the present study was compatible with a study reported from Addis Ababa (Zewdie *et al*., 2016). Higher proportion of T3-ETH sublineage implied its greatest transmission rate in the area. Unlike other studies (Bedewi *et al*., 2017; Alelign *et al*., 2019), it was also found that less number of isolates (2 or 3) with the same spoligotyping patterns were found in a single cluster of this finding. Of course, the less number of isolates in a cluster agrees with a study report from Gambella region, Southwest Ethiopia (Asebe *et al*., 2015). The higher proportion of over all new spoligotyping patterns (86.5%) and new EPTB patterns (93.3%) was against similar studies in Dessie by Birhanu *et al*., (2014) 60.8% which might be due to sample size difference and the type of TB patients understudy in which only EPTB was involved in the case of the later one. The higher proportion of orphan could be due to lack of a report to the spoligotype data base. On the other hand, the leading shared strain type frequently reported from Ethiopia namely SIT149 (Getahun *et al*., 2015; Tilahun *et al*., 2018; Tulu and Ameni, 2018 and Haile *et al*., 2020) was relatively detected at greater rate in this finding.

The highest discriminatory power of the 24-loci MIRU-VNTR to spoligotyping agrees with many other similar studies (Mulenga *et al*., 2010; Biadglegne *etal*., 2015; Bedewi *et al*., 2017;

Shi *et al*., 2018). This implies that the technique has best performance in accurately detecting specific strains of the *M. tb.* It can be used alone and in combination with spoligotyping for the discrimination purpose. Missing some of the 24-loci in this study might be due to amplification problem in the PCRs as similar to the validation of 24-Locus Variable-Number Tandem-Repeat Typing for *Mycobacterium tuberculosis* (Beer *et al*., 2014).

The lowest clustering rate by spoligotyping and absence of clustering at all in MIRU-VNTR of this study is an indication for minor or no recent transmission of the *Mycobacterium* in the area implying the disease is mainly due to endogenous reactivation of the latent TB infection. Similarly, other studies showed that the clustering rate of MIRU-VNTR is less than that of spoligotyping (Ahmed *et al*., 2014; Bouklata *et al*., 2015; Mekonnen *et al*., 2018; Caleffi-Ferracioli *et al*., 2018). In fact, those clustering rate differences in different study areas might be due to the differences in the geography, population density and socio-economic diversity (Pareek *et al*., 2013).

In agreement with previous studies, the predominance of lineage 3 (Delhi/CAS; 32.1%) using MIRU-VNTR 24-loci genotyping showed its wide distribution throughout the country (Tessema *et al*., 2013; Yimer *et al*., 2015; Tadesse *et al*., 2017). In the contrary, it was not the predominant strain rather H37Rv like and Ethiopia_3 were the most common sub-lineages in studies from prisoners and communities in Southern, Southwestern and Southeastern Ethiopia (Ali *et al*., 2016) and Eastern Ethiopia (Mekonnen *et al*., 2018), respectively. This revealed that the predominant lineage of *Mycobacterium* tuberculosis across the country is not well established.

6.3. GenoTypic and Phenotypic Drug Sensitivity Tests

The drug resistant specimens identified by GeneXpert were also confirmed as resistant by both MTBDR*plus* assay and BACTEC MGIT 960 tests. The additional detection of one more drug resistant isolate might be due to single detection of drug resistance by Xpert (only RIF resistant cases). The GenoTypic LPA can detect an additional INH resistance to GeneXpert where as the phenotypic MGIT can detect even more additional drugs such as STM, EMB and INH. This could increase the chance of detecting drug resistant isolates.

The lower proportion of MGIT sub-culture positivity detection from LJ-culture positive *Mycobacterium* sample than the heat killed LPA isolates might be due to the existence of bacterial DNA in the later one. In the case of freezed live *Mycobacterium*, its survival could be interrupted through time until it was sub-cultured by the MGIT DST. The higher sensitivity result 94.2% of LPA and its lower specificity value 30.2% is comparable with similar settings done in Kenya where its sensitivity and specificity was 99.2 and 26.9%, respectively (Aricha *et al*., 2019). This showed that LPA has a good performance in detecting the true positivity of *Mycobacterium* and in a fair agreement (Kappa = 0.276; $P < 0.001$) with the BACTEC MGIT 960 performance in this study.

The overall genotypic drug sensitivity test in this study is higher than other similar studies in the country (Tamrat, 2016; Omer *et al*., 2017; Bekele *et al*., 2018). In contrast, this finding is in less proportion of drug resistance than other study reports from northwest Ethiopia (Alelign *et al*., 2019) and southwest Ethiopia (Tadesse *et al*., 2016). Studies from other countries Chad (Diallo *et al*., 2017), Uganda (Sanchez-Padilla *et al*., 2013), city of Nairobi (Ndung'u *et al*., 2012) and Punjab state of India (Rufai *et al*., 2014) reported greater proportion of resistance. The possible explanation for these variations could be due to difference in sample size, study sites and the study participants.

The rate of MDR in the present study 8.4% (8/95) by genotypic MTBDR*plus* assay is greater than 6.7% (11/165), 1.1% (3/279), 3.1% (5/161) and 1.8% (2/111) of similar findings by Addo *et al*., 2017, Omer *et al*., 2017, Bekele *et al*., 2018 and Alelign *et al*., 2019; respectively. On the contrary, our MDR finding is less than 25.8% (72/279), 17.5% (33/189) and 27.7% (31/112) by Rufai *et al*., 2014; Aung *et al*., 2015 and Tadesse *et al*., 2016, respectively. Regarding the detection of RIF and INH mono-resistance, the current prevalence is in line with a number of studies (Adane *et al*., 2015; Desikan *et al*., 2017; Maningi *et al*., 2017; Mehari *et al*., 2019) that showed rifampicin mono-resistant as greater than isoniazid mono-resistant with varying degrees of proportion. Such RIF resistance is an important implication for higher risk of multi-drug resistance as it is a surrogate marker of MDR (Hillemann*et al*., 2007 and Aung *et al*., 2015). Nevertheless, there are also studies that give us evidence for isoniazid mono-resistance as the leading mono-resistant than RIF (Omer *et al*., 2017; Tilahun *et al*., 2018; Alelign *et al*., 2019).
The greater proportion of TB samples from DRH than the remaining 7 sample collection site is due to the flow of patients to this referral hospital. The findings of all the samples as drug resistant from BMH are because of it's designated as referral hospital for drug resistant patients in northeastern Ethiopia.

Similar to LPA, the phenotypic BACTEC MGIT 960 also detected drug sensitivity to at least one of the four drugs (STM, INH, RIF and EMB) at varying degrees of proportion with other relevant studies. The 15.9% (11/69) drug resistant detection rate in this study was at greater proportion than similar studies done by MGIT (Calver *et al*., 2010; Maru *et al*., 2015; Damena *et* *al*, 2019). On the other hand, lower proportion of drug resistances were reported in this study than other research reports by using the same detection system (Abebe *et al*., 2012; Seyoum *et al*., 2014; Singh *et al*., 2016; Mekonnen *et al*., 2018).

Most of the isolates detected as resistant by LPA were also resistant by MGIT. In two of the samples which were detected as resistant by MGIT but not by LPA was due to its extra drug sensitivity detection rate of streptomycin and ethambutol. All the MDR resistant samples by MGIT in this study were not restricted to isoniazid and rifampicin, but had an additional resistance to EMB, STM or both. This implies that sensitivity detection by MGIT posses an extra benefit to detect more first line TB drugs.

The highest sensitivity of the MTBDR*plus* assay (100%) in detecting RIF resistance in this study is similar with findings from other related studies in Southern (Wondale *et al*., 2018) and Northwestern (Tessema *et al*., 2012) Ethiopia, but with little variation in the isolates from central (Omer *et al*., 2017) and MDR-TB patients referred to the National TB Reference Laboratory (NTRL) of the country (Meaza *et al*., 2017). The specificity of the assay (98.3%) in detecting RIF resistance is comparable to previous report 100% by Tessema *et al*., 2012; 99.8% by Omer *et al*., 2017 and 99.2% by Wondale *et al*., 2018. There is greater variation of MTBDR*plus* assay sensitivity to INH in our study 75% and a study from the Southern Ethiopia 33.3% (Wondale *et al*., 2018). The sensitivity of the assay to INH was 82.7% in a study of isolates from central (Omer *et al*., 2017) and 91.7% in isolates collected from northwestern (Tessema *et al*., 2012) and NTRL (Meaza *et al*., 2017), each. Although the assay's sensitivity result to detect MDR have some discrepancy, the susceptibility result accounts 100% which coincides exactly with other studies reported from the country (Tessema *et al*., 2012; Meaza *et al*., 2017; Omer *et al*., 2017 and Wondale *et al*., 2018).

The study also found that there was an excellent agreement between BD BACTEC MGIT 960 and MTBDR*plus* assay in detecting RIF and MDR with a Kappa value of 0.925 and 0.901, respectively. However, it was found as a good agreement (Kappa value, 0.774) between the two testing methods in detecting INH. Similarly, it was also reported as an excellent agreement in detecting MDR (Kappa value, 1) but with good (0.663) and moderate (0.494) agreement in detecting RIF and INH resistance, respectively (Wondale *et al*., 2018). Such difference between the two findings might be due to the difference in number of drug resistant isolates. Further, the agreement between Culture MGIT and LPA in detecting *katG*, *inhA* and both (*katG* and *inhA*) resistance was almost perfect and agrees with a study by Aricha *et al*. (2019).

6.3.1. Gene mutations associated with rifampicin and isoniazid resistant isolates by GenoType MTBDR*plus* assay

The wild-type *rpoB* probe hybridization band pattern showed an omission of the bands at WT3, WT4, WT6, WT7 and WT8 probes. Surprisingly, a miss of WT8 probe predominates and accounted for 71.4% (10/14) among the RIF resistant isolates in a small region of amino acids located between position numbers 530-533 of the *rpoB* gene. Omission of all the *rpoB* gene probes was without any gain of probes in the MUT region and depicted as "unknown" mutation. Such lack of binding of a WT probe without simultaneous binding of a mutant probe is likely caused by the presence of a resistant mutation.

Similarly, a report from central Ethiopia in line with our finding no gain of any mutant band was identified for the non-hybridized WT8 probe (Omer *et al*., 2017). Other findings were also reported for the absence of MUT band from different countries (Brossier *et al*., 2006; Huyen *et al*., 2010; Singhal *et al*., 2012; Mohan *et al*., 2014; Addo *et al*., 2017). Despite such reports, there were research findings where gain of MUT probes were identified in a part of the *rpoB* gene (Biadglegne *et al*., 2013; Seifert *et al*., 2016; Tadesse *et al*., 2016; Prasad *et al*., 2019).

Greater frequency of resistance 8/16 (50%) to INH occurred due to mutation of the *katG* gene, whereas lower frequency of resistance 5/16 (31.3%) was caused by the mutations in the promoter region of the *inhA* gene. Hybridization that forms a band was missed in both *katG* gene and *inhA* promoter region in one isolate. Greater frequencies of the *katG* gene WT omission at codon 315 was also reported from other studies in Ethiopia that agrees with this finding (Tadesse *et al*., 2016; Brhane *et al*., 2017; Omer *et al*., 2017; Alelign *et al*., 2019). A miss of WT probe at Codon 315 in the *katG* gene without the presence of specific MUT band accounted for 75% (6/8) in this study. The remaining 25% of the strains had mutations in the *katG* gene at codon 315 with amino acid change of S315T1 (AGC→ACC). No mutation was identified at MUT2 probe of the LPA strip. The *inhA* promoter region showed a miss of -15/-16 codon that was detected in 5/16 (31.3%); and 1/16 (6.3%) at -8 gene region without conferring any addition of specific mutational band in both cases. This mutation also agrees with the finding from central Ethiopia and also considered as 'unknown' mutation (Omer *et al*., 2017).

6.3.2. Genetic diversity of the drug resistant *Mycobacterium tuberculosis*

The higher proportion of Euro-American lineages among drug resistance strains could be due to its higher prevalence under the interpretable spoligotyping results of the isolates done using LPA in this study. This is in line with the findings that reported greater proportion of the same major lineage from different parts of Ethiopia (Getahun *et al*., 2015; Molina-Moya *et al*., 2017; Tulu and Ameni *et al*., 2018; Alelign *et al*., 2019). Based on the detection of their geographic distribution the majority 82.4% (14/17) of the drug resistant isolates were identified as orphans and have no SIT number in the SITVIT2 data base. The most predominant families identified among the drug resistant isolates include the T and Manu families which is similar to different studies on genotyping and drug resistance patterns of isolates (Bazira *et al*., 2011; Mihret *et al*., 2012; Esmael *et al*., 2014). The clustered strains of the study also showed higher frequency and a statistically significant association with any anti-TB drug resistance than the unique ones revealing an implication for the highest risk of drug resistance among recent TB transmission. This is consistent with a study in Russia that found higher incidence of drug resistance in clustered strains of *Mycobacterium* (Toungoussova *et al*., 2002).

6.4. Assessment of Nutritional Status

Several conditions including undernutrition, smoking and co-infections are important risk factors that aggravate the progress of TB (Dooley *et al*., 2009; Lonnroth *et al*., 2009). To this effect, our assessments of nutritional status among TB cases using BMI showed about half of the cases were under chronic energy deficiency. Similar studies in other two areas of Ethiopia; Addis Ababa and Arba Minch found lower proportion as compared to the present finding which might be due to the difference in time of taking the measurements (Dargie *et al*., 2016; Alemu and Mama, 2017). In this study, measurements were taken on the spot of TB screening period when the patients' body weight is expected to be low. However, the measurements of body weight were taken during the follow up of drug therapy both in Addis Ababa and Arba Minch study cases when their body mass might be improved due to the follow up treatments. In fact, studies in Ghana and urban Tanzania reported comparable prevalence of undernutrition to this finding in *M. tb*parasitic co-infected patients. In all indicated studies, mild malnutrition was at its highest peak than moderate and severe malnutrition (Dodor *et al*., 2008; Dargie *et al*., 2016; Mhimbira *et al*., 2017).

The higher percentage of undernutrition in *M. tb* parasitic co-infected cases in this study than parasite free TB cases could imply that co-infection had an impact on the nutritional status of TB cases. Measurements using MUAC also confirmed the findings of BMI in similar way that greater percentage of the TB cases with parasitic co-infection were undernourished than those of non-coinfected cases. Such greater rate of undernourishment in co-infected cases might be due to the parasitic impact. Similar finding of nutritional assessment using both BMI and MUAC parameter suggests co-infection have an impact on the nutritional status of TB cases (Abate *et al*., 2015; Alemu *et al*., 2019). In addition, implication of nutritional status difference among TB cases and the control group using both BMI and MUAC may be attributable to tuberculosis. The weight loss in this study agrees with the clinical symptoms criteria for screening tuberculosis cases (Berg *et al*., 2015).

6.5. *Mycobacterium tuberculosis* -Parasite Co-infections

In this study, it has been observed that as family size increased the risk of TB transmission in the community also increased. This might be due to the high risk of contacts among family members. Higher rate of infection in farmers and those who cannot read and write might be due to lack of awareness about the disease prevention, immediate diagnosis and treatment (Alemayehu *et al*., 2014; Alemu and Mama, 2017). should be under KAP!

Prevalence of *M. tb*-parasitic co-infection in this study is less than the recent co-infection rate 22% (20/91) reported from Addis Ababa (Alemu *et al*., 2019). This is also true with the higher

M. tb intestinal parasitic co-infection prevalence rate reported from other parts of Ethiopia in Gondar 33.3% (24/72) and Arba Minch 26.3% (56/213) (Alemayehu *et al*., 2014; Alemu and Mama, 2017). These higher co-infection rates in other study sites of Ethiopia as compared to the present finding might be due to the difference in geographical settings and the study participants. The age group of the study participants also vary in that only those who were 18 years and older were considered in this study whereas in the case of the former studies those who were younger than 18 were included. Previous study in the country revealed that lower age groups possess higher parasitic infection than the older age groups (Mengistu *et al*., 2014). As there is no gold standard test for parasitic stool examination, differences in the testing methods (direct saline, SAF and Kato-Katz) might also be another factor for the variations of TB parasitic co-infection since the sensitivity of one diagnostic method is different from the other (Endris *et al*., 2013).

Similar to this study, findings in Arba Minch reported that the prevalence of intestinal helminth infections was greater 24.4% (52/212) than that of intestinal protozoa 6.1% (13/212) (Alemu and Mama, 2017). Comparable studies on *M. tb*-parasitic co-infection in northwestern Ethiopia also reported higher helminthic co-infection than protozoa (Alemayehu *et al*., 2014). Although helminthic infection is high in both of the studies, their percentage varies as compared to the present finding which might be due to the difference in study subjects as well as the study sites and other factors. Contrary to the overall high prevalence of *A. lumbericoides* infection in the country, the present finding showed *S. mansoni* to be the most widespread helminthic infections followed by *A. lumbericoides*. This is perhaps due to the high prevalence and endemicity of schistosomiasis in Oromia Special Zone specifically in Bati and Kemise areas of the study site (Deribe *et al*., 2012; Mama and Alemu *et al*., 2016).

Although there was no significant difference in parasitic co-infections among male and female study participants, males were infected in a greater proportion than females similar to the former finding in northwest Ethiopia (Alemayehu *et al*., 2014). By contrast, a report from China showed that females are 2.05 times more likely to acquire IPIs than males (Li *et al*., 2014). Such differences between Ethiopia and China might be due to larger number of males than females in study from China.

Culture positivity, pulmonary and extra-pulmonary tuberculosis

The less proportion of culture positivity in this study is close to the study in Addis Ababa (Adane *et al*., 2015) which might be due to the delay of culturing time, electric interruption when the specimens were preserved in the refrigerator at the sample collection sites and long distance travel from temporarily stored collection site to ALIPB where the specimens were cultured. These factors increase the chance of bacterial death in the collected sputum samples. Perhaps, it could also be the expectation that bacterial culture positivity might decrease when the samples were stored in the refrigerator for a long duration than instant culturing. On the contrary, most studies reported that culture positivity was at large proportion (Debebe *et al*. (2014); Kadioglu *et al*. (2014); Asebe *et al*. (2015); Bedewi *et al*. (2017)).

The extent of pulmonary and extra-pulmonary TB culture positivity in this study is lower than that of a study in India (Sinha *et al.* 2016). A study from different sites in Ethiopia also reported greater culture positivity of both clinically manifested smear positive pulmonary 79% (753/953) and extra-pulmonary 38% (456/1198) TB (Berg *et al*. 2015). Likewise, a study report from northwestern Ethiopia and Addis Ababa showed greater culture positivity of PTB than EPTB (Korma *et al*., 2015; Fanosie *et al*., 2016). In all instances, there is less culture positivity of

EPTB than pulmonary ones which might be due to cytological suspicion of the specimen by pathologist unlike to the detection of disease causative organism itself as in the case of PTB. Moreover, the suspected *Mycobacterium* cellular infection could be paucibacillary which decrease the sensitivity of diagnostic test in EPTB.

The level of EPTB in Ethiopia is much higher (33%) than the global average (15%) (Berg *et al*., 2015). The finding of this study also agrees with the global report that the proportion of EPTB is high in Ethiopia which might be due to diagnostic challenges including shortage of pathologists to identify and treat the cases on time in most health institutions. There was no any pathologist to diagnose EPTB in all governmental health institutes including the referral hospital where this study was done. Because of this, all the suspected cases were referred to a single private diagnostic laboratory (BHDL) and remained as the diagnostic challenge of the area.

The EPTB has different manifestations based on the organs to be attacked and its intent of dissemination in the body. Similar to other relevant studies in the country, this finding also revealed that lymph nodes as the leading organs affected. In fact, the percentage of their infection rate differs as cervical, auxiliary, inguinal, supra-clavicular, sub-mandibular and anterior neck lymph nodes (Berg *et al*., 2015; Korma *et al*., 2015). The higher infection of lymph node is similar to the study reported from Germany (Forssbohm *et al*., 2008). On the other hand, the most common sites involved were bones/joints and lymph nodes in United States of America (Peto *et al*., 2009), whereas the genitourinary system and skin were the common sites of infection reported from Hong Kong (Noertjojo *et al*., 2002). Such differences might be attributable to either host or pathogen related factors as well as access to patient sample collection in the clinical settings.

Cervical lymph nodes were found to be the most frequently infected anatomical site similar to other report from Ethiopia (Biadglegne *et al*., 2013). This higher infection rate might be due to the physical proximity of lymph node to the route of infection where the bacilli can easily be picked up by macrophages or dendertic cells that facilitate the transportation of the bacilli in the cervical lymph nodes causing pathology. It could also be expected that the bacteria could easily spread from intra-thoracic lymphatics to the cervical areas.

6.6. Tuberculosis Patients Awareness towards the Disease

Assessment of knowledge, attitude and practice is vital for the implementation of TB prevention and control program. Although majority of the TB patients in this study heard about TB, it is in less proportion as compared to high school students in southern Ethiopia (Hibstu and Bago, 2016) which implies students have a great opportunity to hear about the disease either through their education at school or through different media.

The lower proportion of TB patients who heard about drug resistant bacteria either through healthcare workers or other sources of information is against a study on university students in Pakistan (Javed *et al*., 2016). The implication of such low proportion to hear about the drug resistance bacteria is that a lot of work is required to create awareness and minimize the alarming drug resistant TB against the "End TB strategy" of WHO in the study area.

Similarly, lower proportion of TB patients in this study knew about the etiologic agent of TB than high school students 81.7% (201/206) in southern Ethiopia (Hibstu and Bago, 2016) which might be due to knowledge gained at school. In addition, lower proportion of study participants determine the correct etiologic agent as bacteria/germ among prisoners in East Shao Zone of Ethiopia (Kura *et al*., 2010), northern (Adane *et al*., 2017) and community participants in central

Ethiopia (Moreda *et al*., 2018) with a proportion of 31%, 37.7% and 34%,, respectively. Also a study report from other countries showed lower proportion of community participants that knew the causative agent of TB in India (Easwaran *et al*., 2015) and among population based study in Lesotho (Luba *et al*., 2019).

The final year university students in Iran (Behanz *et al*., 2014), community studies in Malaysia (Salleh *et al*., 2018) and patients at primary health care in South Africa (Kigozi *et al*., 2017) detect the right etiologic agent of TB with greater percentage than the present finding as 92.9% (130/140), 88.2% (90/102) and 60.2% (305/507); respectively. The overall finding variations from different study reports showed educated participants might have more knowledge to tell the exact causative agent of TB. Contagious nature of the disease and its curability was well known by majority of the TB patient participants which in line with other relevant studies (Kenyi *et al*., 2014; Hibstu and Bago, 2016; Kigozi *et al*., 2017; Luba *et al*., 2019) which might be due to the circulating nature of TB among the communities.

The study patients in the current study who checked for TB on time is in less proportion to 66.7% (68/102) a study in Malaysia (Salleh *et al*., 2018) and India 76.9 %(1563/2032) (Easwaran *et al*., 2015) which might be due to lack of awareness. Majority of the TB cases in this study were not aware of the likelihood of being infected by drug resistant strains comparable to study prisoners in North Ethiopia (Adane *et al*., 2017) implying drug resistant TB is relatively unknown. In addition, most of the TB patients didn't know the required treatment duration for drug resistant TB in contrast to the drug resistant patients who knew the correct treatment duration. Such differences could be due to awareness disparity between drug resistant TB patients and the other form of TB cases (Dzeyie *et al*., 2019).

7. Conclusion and Recommendations

Both pulmonary and extra-pulmonary TB were commonly occurring in northeastern Ethiopia. Majority of the TB patients included in the present study were undernurished and also co-infected with intestinal parasites. The percentage of culture positivity was low and all of the isolates were *M. tuberculosis*. The genetic diversity of the isolates was high and about 80% of the isolates were classified as single strains (singletons) while only 20% of the isolates were grouped under clustered strains. Furthermore, the 56 isolates typed by MIRU-VNTR wereall classified as a single strain. The percentages of mono-drug resistance and multi drug resistance were relatively high in the northeastern Ethiopia. On the basis of this conclusion, the following recommendations were forwarded:

1. Strengthening the TB control Program of the study area (Oromia Special Zone and South Wollo Zones),
2. Public education on proper use of anti-TB drugs and TB transmission,
3. Proper monitoring of MDR TB cases and controlling its further transmission
4. Sustainable improvement of the nutrition requirement of the community,
5. Regular de-worming of the community in the area,
6. Public education on the sanitation procedures, importance of nutrition and related health issues,

Areas of future study

This study was conducted by collecting samples from some health institutes of South Wollo and Oromia Special Zone and did not include all the districts. Thus, it could not be a representative of the whole geographic area of northeastern Ethiopia. Further studies of molecular epidemiology and drug sensitivity patterns of the *Mycobacterium* could be conducted to identify the novel strains available in the area by culturing positive samples on the spot at the currently established regional laboratory in Dessie Town. The findings of all the strains as unique and orphans using MIRU-VNTR 24-loci also needs further investigation with large number of isolates.

Limitation of the study

1. Less number of culture positivity from large smear positive samples collected due to various reasons.
2. Although repeated trials of 24-loci MIRU-VNTR was done in this study on heat killed isolates of LJ culture positive, all of them were negative therefore, the study was conducted only on MGIT 960 positive isolates.
3. Drug sensitivity profile was not done for the second line drugs among the identified drug resistant isolates.
4. Examination of a single faecal specimen, faecal examination was not done for 32.5% of TB patients, sputum and faecal examination was not done for the control groups and that a small number of participants fulfilled our matched criteria. All these might have a potential bias for the study.

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9. Annex

Appendix I. Participant information sheet

Dear participant:

Project title: **Molecular Epidemiology, Drug Sensitivity Patterns, Parasitic co-infections and associated factors of *Mycobacterium tuberculosis* in Amhara Regional State, Northeastern Ethiopia**

Background: Tuberculosis is an air borne disease caused by acid-fast bacterial agent called *Mycobacterium tuberculosis complex*. Usually, the bacteria infect the lung while other body parts can also be infected in rare cases as extra-pulmonary tuberculosis. Despite advances made in understanding the pathogenesis and management of this disease, *M. tb* infects about one third of the global population. The disease is found throughout the world but more common in those countries with low socioeconomic status, malnutrition, overcrowding population, low knowledge of people for early symptoms, low infrastructures and absence of nearby health facilities, and scarcity of skilled health professionals. Besides, the bacterial infection is dominant in economically most productive age groups (15 to 54 years). Lack of awareness to be tested at early stage and inaccurate treatment made this curable disease as hazardous to drug resistant ones. On the subject of this bacterial infection, Ethiopia is ranked as the fourth from Africa and one of the top 30 high TB burden countries globally. There were no established knowledge on the molecular epidemiology and transmission dynamics of the bacteria in the country which have a considerable role in the prevention and control of tuberculosis. In this study, Oromia Special Zone and South Wollo Zone were selected to investigate the *Mycobacterium* diversity, drug sensitivity pattern, prevalence of intestinal parasitic infections among TB cases and awareness toward the disease.

Objective of the study:

The general objective of this study will be to investigate molecular epidemiology, drug sensitivity profiles and associated factors of tuberculosis in northeastern Ethiopia.

Procedure: The study participants will be enrolled after consent was obtained based on the inclusion criteria set. You are screened as tuberculosis case by health physicians of the health center and based on the prepared suggestive questionnaire. Of those patients you will be asked for knowledge, attitude and practice. In the health centers, smear positive TB patients will be enrolled for the study and requested to provide about 3-5ml sputum on the spot for this research work. The collected positive sputum test will be taken to ALIPB for further analysis of molecular epidemiogy and drug sensitivity pattern. Some of the laboratory procedures might not be performed within the country. Therefore, samples would be shipped to laboratories outside Ethiopia. Beside, direct stool test will be done on the spot for identification of parasitic co-infection among smear positive participants. Kato-Katz will be prepared to determine the intensity of eggs per gram in the stool and SAF concentration techniques to detect the parasites even in light infection.

Risks associated to the study: You may feel some minor pain and discomforts, while body fluid is drawn from the EPTB suspected sites of your body. But this pain will disappear after a few hours. In other cases providing sputum and stool have no any risk. In all the steps, an experienced health professional will carry out the procedure with standard aseptic and sterile conditions. For any inconveniences related to these procedures, you will be provided appropriate medical care.

Benefits to the study participants: you will know your health status with respect to tuberculosis, parasitic infection and nutritional status. But you have unconditional right to refuse to be part of this project. In case of TB drugs need, you will get the standard care available and treatment with proper guideline and counseling for treating patients from the health center. For parasitic helminth infection the cost of drug will be covered by this research project.

Confidentiality: Any information that will be collected from you during this research will be kept confidential. Your answers to the questioners will be registered on questionnaire paper. Your name will not be mentioned in any report and the result of the study will be used for scholar purposes only.

Sharing results:The results of this study will be disseminated through publications or other means to be accessed by scholars, health physicians and tuberculosis concerned bodies. In addition the findings will be submitted or presented to Oromia Special Zone and South Wollo Zone health bureau of Amhara region.

Rights to refuse or withdraw: - Your participation should be voluntary. You could choose not to participate. If you decide to participate, you may have also full right to withdraw at any time without any preconditions. Even if you do not want to take part in this study, you will still be able to be treated at this centre according to the usual standard of care and will not lose any benefits. Do you understand what has been said to you? If you have any question you have the right to get proper explanation.

Contact Address: In case of any question regarding the study or related issues, you can contact the following individuals;

1. **Mr. Fikru Gashaw**: College of Natural Sciences, Addis Ababa University, POBOX 1176 Addis Ababa Ethiopia , Telephone (mobile): +251-911-145506, E-mail: fikrug2012@gmail.com
2. **Prof. Gobena Ameni**: Aklilu Lemma Institute of Pathobiology, Addis Ababa University, POBOX 1176 Addis Ababa Ethiopia, Telephone (mobile): +251-911-413073 E-mail: gobenachindi@yahoo.co.uk
3. **Prof.Yalemtsehay Mekonnen:** College of Natural Sciences, Addis Ababa University, POBOX 1176 Addis Ababa Ethiopia , Telephone (mobile): +251-913-244396, E-mail: yalemtshay.mekonnen@aau.edu.et
4. **Prof. Berhanu Erko:** Aklilu Lemma Institute of Pathobiology, Addis Ababa University, POBOX 1176 Addis Ababa Ethiopia, Telephone (mobile): +251-911-371173 E-mail:berhanue@yahoo.com

Thank you for your kind cooperation and participation in this study!

Appendix II. Informed Consent Form

Identification No: ______________________

Date: ________________________________

Dear Participant, you are invited to participate in this tuberculosis research project for the Doctorate's of the researcher. The purpose of this research is to investigate molecular epidemiology, drug sensitivity patterns and associated factors of tuberculosis which is expected as having an input for tuberculosis control program in this area. The project has been approved by college of natural science, AAU and gets permission from Amhara region Oromia Special Zone and South Wolo Zone health bureau. Your participation should be voluntary. You have the right not to participate. If you decide to participate, you may also withdraw at any time without any preconditions. The information that you will provide us is confidential to our best. The result of this study will be used for scholar purposes only.

Please read, tick off each of the boxes and sign the form if you agree to take part in this study.

1. I understand what this study is about and know how to contact the investigators if I want to. ☐
2. A portion of the sample may be stored and used for this study only. If there is leftover after the completion of the study, it will be discarded safely. ☐
3. A portion of the Heat-killed cells will be shipped abroad for further analyses. ☐
4. I understand that all the information given to the investigators and all test results will be kept private and confidential. ☐
5. I understand that I will not get benefit financially from this study except treatment drugs for parasitic helminth infections. ☐
6. I understand that I am free to withdraw myself from this study if I want to. ☐
7. I understand that if I refuse to take part in this study my care will not be affected. ☐

I have been given enough time to think over before I signed this informed consent. It is, therefore, with full understanding of the situation that I gave my informed consent to participate in this study.

The information was explained to me by: ____

Name of participant (guardian): __________________________ Signature:- _________________

Name of the physician: ____________________________ Signature:- _________________

ተጨማሪመረጃ II.የስምምነትቅጽ

ቀን______________

መለያቁ. ______________ዕድሜ______________

ውድተሳታፊያችንበዚህበሳንባነቀርሳበሽታየዶክተሬትጥናትላይእንዲሳተፉተጋብዘዋል፡፡

የምርምሩአላማምየሳንባነቀርሳበሽታንየሚያስከትልየባክቴርያዝርያ (ዘረመል)

ለይቶስርጭቱንናተያያዥነትያላቸዉንነገሮችማየትነዉ፡፡

ይህፕሮጀክትበአአዩበተፈጥሮሳይንስኮሌጅጸድቆበአማራብሔራዊክልልየኦሮሚያልዩዞንደቡብ

ወሎጤናመምሪያናበወረዳዉጤናቢሮፍቃድአግኝተዋል፡፡

ተሳትፍዎበእርስዎፈቃደኝነትላይየተመሰረተናላለመሳተፍምሙሉሙብትአለዎት፡፡

ለመሳተፍከወሰኑበኋላምበፈለጉትጊዜካለምንምቅድመሁኔታመዉጣትምይችላሉ፡፡

የሚሰጡንትክክለኛመረጃምለጥናቱከፍተኛአስታዋጽኦአለዉ፡፡የዚህጥናትየምርምርዉጤትምለምርምርሥራዉብቻይውላል፡፡

እባክዎትንአንብበዉ (ቱነበልዎት) ☐

በጥናቱለመሳተፍፈቃደኛከሆኑበሳጥኑውስጥምልክትአድርገዉበዚህየስምምነትወረቀትላይይፈርሙ፡፡

1. ይህጥናትምንእንደሆነተረድቼተመራማሪዎቹንምለማግኛትብፈልግምእንዴትእንደማገኝአውቃለሁ፡፡
2. የምርምርናሙናውተሰብስቦለዚህጥናትብቻያገለግላል፡፡ከምርምሩየሚተርፍካለምበጥንቃቄይወገዳል፡፡ ☐
3. የተወሰነናሙናለተጨማሪየምርምርስራወደዉጪሀገርይላካል፡፡ ☐
4. ለተመራማሪዎቹየተሰጡትመረጃዎችናየምርምርውጤቶችምስጢራዊነታቸዉእንደሚጠበቅተረድቻለሁ፡፡ ☐
5. ለጥገኛህዋሳትከሚሰጠኝመድሃኒትዉጪሌሎችየገንዘብጥቅማጥቅምእንደማላገኝተረድቻለሁ፡፡ ☐
6. በፈለግኩኝጊዜጥናቱንማቋረጥእንደምችልአውቃለሁ፡፡ ☐
7. በዚህጥናትባልሳተፍምበማገኛዉየህክምናአገልግሎትላይተጽእኖእንደማያመጣብኝተረድቻለሁ፡፡ ☐

ይህንንየስምምነትመረጃከመፈረሜበፊትእንዳስብበትበቂጊዜተሰጥቶኛል፡፡

በዚሁመሰረትየጥናቱንሁኔታበሙሉተረድቼበጥናቱለመሳተፍየስምምነትቃሌንሰጥቻለሁ፡፡

ይህን መረጃ ያብራራልኝ ---

የተሳታፊዉ (ያሳዳጊ) ሙሉስም --ፊርማ፡ --------------------

የሀኪሙስም --- ፊርማ፡ ----------------------

Odeeffannoo Dabalataa II. Uunkaa Odeeffanno Waligalte

Lakkofsa addaa: ________________________ Umuri ________________

Guyyaa: ____________________________

Kabajamoo hirmaata/tu, qoranno doktareetii kan ta'e wa'e dhibee sombaa kanarratti akka hirmaattaniif afferamtanii jirtu. Kaayyon qoranichaas tatamisa'uu fi rakoowwan dhimmoota kanaan walqabatan caasaa dhukkuba TB ittisuuf kan gargaaranii dha. Projaktiin kun kolleejjii uumama saayinsii yunivarsitii finfineetiin mirkanaa'ee, naannoo Amaaraa godina addaa oromiyaatti aanaa kanaraa eyyama argatee jira. Hirmaanan keessan fedha/eeyyama keessanrratti kan hundaa'e ta'uu qaba. Irratti hirmaachuufis ta'ee dhiisuf mirga qabdu. Hirmaachuf yoo murteesitanis yeroo feetan keesaa ba'u ni dandeesu. Yaadini issin nuuf kennittanis dhugaa irratti hundaa'u qaba. Xumurri qorannoo kanaa hojii barumsaa qofaaf oola.

Mee dubbisaa, yoo fedhii keessan ta'e saanduqoota keessatti sararaa. Dabalataanis qoranno kana keessatti hirmaachuu keessaniif malateesaa.

1. Waa'en qorannoo kanaa nuuf galee jira. Warra qoratannis yoo barbaane akkamtttii akka argachu dandeegnu beekne jira. ☐
2. Fakkisa (sample) amita'e kuusamee qorannoo kana qoffaaf oola. Qorannoo kana irraa kan hafee amoo eegumsi godhameefii ofiegganoodhan dhabamsiisama. ☐
3. kan kuusame irraa ammi ta'e immoo qorannoo gadi fageegnaf gara biyya alaatti ergama ☐
4. Odeffannoonii fi bu'aaleen qorannoo warra qorattoota kanaaf kennaman iccitiin isaanii akka eegaman hubadheen jira. ☐
5. Qoricha dhibee maxanituu raammoo (parasitic helminthes) naa kennamuun allatti qorannoo kana irraa faayidaa qarshii akkan hin arganne hubanee jira. ☐
6. yeroo barbaanes qorannoo kana keessaa ba'u akka dandeenyu beekne. ☐
7. qorannoo kana irratti yoo hirmaachu baannes deeggersi manni yaalaa kun nuuf taasisu irratti dhiibbaa akka hin qabaannee nuuf galee jira. ☐

Waligaltee kana odoon hin mirkaneesin dura akkan itti yaaduf yeroo ga'an naaf kennamee jira. Kanaaf waligalteen kun sirriti naaf galeeto qorannoo kana keessatti hirmaachuf nan mirkaneesa.

Odeeffannoo kana kan naaf ibse: ____

Maqaan hirmaaticha (bakka bu'aan daa'imaa) ____________________ mallattoo: _______

Maqaan nama yaaluu: ______________________________ mallattoo: ____________

Date ________________

Appendix III. Questionnaire

Dear participant, this is a PhD research project to determine molecular epidemiology, drug sensitivity patterns and associated risk factors of tuberculosis (TB) in Amhara national regional state of Oromia Special Zone, northeastern Ethiopia. Drug sensitivity patterns of the bacteria and the local participants' knowledge, attitude and practice toward the disease will be assessed. In addition, the prevalence of parasitic co-infection to TB will also be measured. It is expected that the study have a great input for the prevention and control program of the diseases in the area. The project got approval from AAU department of Microbial, cellular and molecular biology. Permission was also obtained from Amhara national regional state Oromia Zone health bureau, the Woredas health office and this health center. Your participation and unreserved response to the following questions have great input for the study. Writing your name on the questionnaire paper is not needed. Finally, I want to express my deepest gratitude for your concern and valuable time to perform the questionnaires activity.

Note: ☞ Mark ✓ in the box where appropriate

☞ Circle the letter of your choice for multiple questions

A. General information

1. Code of participant: ____________ Region: Amhara, Zone: ______________________, Woreda:_______________________Kebele:_____________________________________
2. Age ___________________
3. Gender Male ☐ Female ☐
4. Marriage Single ☐ Married ☐ Divorced ☐
5. Family size ______________
6. Ethnics _____________
7. Religion ________________
8. Educational level
   a. No school b. Elementary c. High school
   d. Higher education - certificate ☐ diploma ☐ degree ☐ MSc and above ☐
   e. Religious schooling
   f. Literacy classes (Adult education)

8. Job description
   a. Government worker b. Merchant c. Farmer
   d. Labor worker
   e. No job
   f. If other specify ___________________________________

B. Basic questions

1. Do you know about the disease tuberculosis? If your answer is ‘Yes’ go to number 2
a) Yes b) No

2. From whom you know it for the first time?
a) Family, friends, neighbors and colleagues b) A patient sick of TB
c) Health workers (like health extension workers and nurses) d) Teachers
e) Media (like Radio and TV) f) Books, brochures, posters and other printed materials
g) Others (please specify) ____

3. Have you ever seen TB patients so far? a) Yes b) No

4. If your answer is ‘yes’ for Q3 where? ____
____

5. Is it contagious? a) Yes b) No c) Don’t know

6. What is the causative agent for TB?
a) Bacteria/germ b) Cold air c) Shortage of food d) Smoking
e) Chewing ‘Khat’ f) Dust h) don’t know
g) If others (please specify) ____

7. TB bacteria/germ attacks
a) Lung only b) Other body part but not lung
c) Both lung and other body part d) Don’t know
e) Others (please specify) ____

8. Is TB a treatable disease? a) Yes b) No c) Don’t know

9. Where do you go first when you feel sick?
a) Traditional healers’ b) Religious beliefs
c) Private clinics
d) Governmental health institutions like health posts, clinics, health center or hospital
e) Clinics run by non-governmental organization
f) Others (please specify)

10. Do you check for TB at health institutions when you have prolonged cough (more than 3 weeks)?
a) Yes b) No c) Sometimes

11. Based on your view, who can be infected with TB?(Please check all that are listed)
a) Anybody b) Only poor people c) Only homeless people
d) Only alcoholics e) Only drug users
f) Only people living with HIV/AIDS g) Only people who chew ‘Khat’
h) Other (please specify): ____

12. How can someone with TB be cured?(Check all that are mentioned)
a) By using herbal remedies (traditional medicine)
b) Taking home rest without medicine

c) By praying
d) By using specific drugs given by health centre based on their prescription
e) Do not know
f) Other (please specify): ____

13. Have you ever been sick of TB so far? a) Yes b) No

14. Which age groups of the society are mostly attacked by TB? Why?

a) Birth to fifteen years ____

b) Fifteen to fifty four years (working age group) ______________________________

c) Above fifty four ____

d) All are equally attacked ____

e) Don't know ____

15. How can a person get TB?(Please check all that are mentioned.)
a) Through handshakes b) Through the air when a person with TB coughs or sneezes
c) Through sharing dishes d) Through blood
e) Through touching items in public places (doorknobs, handles in transportation, etc.)
f) Through sexual intercourse
g) Do not know
h) Other (please specify): ____

16. Do you cover your mouth at the time of coughing and while others are coughing near you
a) Yes b) No c) Some times

17. Have you ever heard drug resistant TB bacteria so far? If your answer is yes go to number 18
a) Yes b) No

18. What makes the bacteria as to be drug resistance ones? (Can choose more than one)
a) Incorrect use of antimicrobial drugs based on the prescription of health workers.
b) Non-adherence of the drugs to the prescribed regimen/natural phenomenon due to mutation
c) Its transmission from those patients who develops drug resistant bacteria.
d) Its transmission from any TB patients.
e) Don't know
f) Others (please specify) ____

19. Drug resistant bacteria took
a) Equal duration as non resistant ones for treatment
b) Less duration than non resistant ones for treatment
c) Greater duration than non resistant ones for treatment
d) Can't be treated
e) Don't know
f) others (please specify) ____

20. How can individuals be safe from TB?

a) By avoiding shaking hands
b) By Covering mouth and nose when coughing or sneezing
c) By avoiding sharing dishes
d) By washing hands after touching items in public places
e) By closing windows at home
f) Through good nutrition
g) By praying
h) Do not know
i) Other (please specify): ____

21. Do you chew ‘Khat’? If so go to Q. 22
    a) Yes   b) No
22. How many times per week in average? ______________________
23. If you consume ‘Khat’ it is
    a) With peers   b) alone
    c) sometimes in peers
24. Chewing ‘Khat’ with peers in congested rooms by closing windows could have more risk for TB transmission.   a) True   b) False   c) I don’t know
25. List some diseases (co-infections) as a result of TB or the reverse (TB as a result of other diseases)?
    ____
    ____
26. Is TB a zoonotic disease?
    a) Yes   b) No   c) Don’t know
27. If yes, how ____
28. List the signs and symptoms of TB?
    ____
    ____
    ____
29. How do you treat TB patients at home? Please explain
    ____
    ____
    ____
    ____

ቀን____________

ተጨማሪ መረጃ III. መጠይቆች

ውድ ተሳታፊያችን ይህ የፒ ኤች ዲ ፕሮጀክት ምርምር የሳንባ ነቀርሳ በሽታ የሚያስከትለዉን የባክቴርያ ዝርያ መለየት፡ መድሀኒት የተለማመዳና እንዲሁም ተያያዥነት ያላቸዉን ነገሮች በአማራ ብሔራዊ ክልል ኦሮሚያ ዞንና ደቡብ ወሎ ዞን ሰሜን ምስራቅ ኢትዮጲያ ለማወቅ ነው፡፡ የባክቴሪያዉ ከመድሃኒት ጋር መለማመድና የአከባቢዉ ማህበረሰብ ዕውቀት፣ አመለካከትና ስለበሽታዉ ያላቸዉን ግንዛቤም ጥናቱ ይዳስሳል፡፡ በተጨማሪም ቲቢ ከጥገኛ ህዋሳት ጋር ያላዉን ቁርኝት በጥናቱ ውስጥ የካተታሉ፡፡ ጥናቱ በአከባቢዉ በሽታዉን ለመቆጣጠርና ለመከላከል ያለዉን እቅድ ለማሳካት ትልቅ ግብዓት ይኖረዋል ተብሎ ይታማናል፡፡ ፕሮጀክቱ ከአአዩ ማይክሮቢያል ሴሉላርና ሞሎኪዩለር ባዩሎጂ ትምህርት ክፍል እውቅና አግኝተዋል፡፡ በተጨማሪም ከአማራ ብሔራዊ ክልል ኦሮሚያ ልዩ ዞንና ደቡብ ወሎ ዞን ጤና መምሪያ፣ ከወረዳዉ ጤና ቢሮና ከዚህ የህክምና ማዕከልም ፍቃድ አግኝተዋል፡፡

ስለዚህ ለሚከተሉት ጥያቄዎች እርስዎ የሚሰጡት ያልተቆጠበ ምላሽ ለጥናቱ ከፍተኛ አስታወጽኦ አለዉ፡፡ በጥያቄ ወረቀቱ ላይ ስም መጻፍ አያስፈልግም፡፡ በመጨረሻም ጥያቄዎቹን ለመመለስ መስዋእት ላደረጉት ግዜዎትና ለጠሰት ትኩረት በጣም እናመሰግናለን፡፡

ማስታወሻ ☞ ይህንን ምልክት ✓ በትክክለኛዉ ሳጥን ውስጥ ያድርጉ

☞ ምርጫ ላላቸዉ ጥያቄዎች ምርጫዎትን የያዘዉን ፊደል ያክብቡ

ሀ. አጠቃላይ መረጃ

1. የተሳታፊዉ ኮድ :_________ ክልል አማራ ዞን ________________

   ወረዳ ________________ ቀበሌ : _________________________

2. ዕድሜ _____________

3. ጾታ ወንድ ☐ ሴት ☐

4. የጋብቻ ሁኔታ ያላገባ ☐ ያገባ ☐ የተፋታ ☐

5. የቤተሰብ ብዛት __________

6. ብሔር __________

7. ሃይማኖት __________

8. የትምህርት ደረጃ

   ሀ) አልተማርኩም ☐ ለ) የመጀመርያ ደረጃ ☐ ሐ) ሁለተኛ ደረጃ ☐

   መ) ከፍተኛ ትምህርት ተቋም - ሰርተፍኬት ☐ ዲፕሎማ ☐ ድግሪ ☐ ማስተርስና በላይ ☐

9. የስራ ሁኔታ

   ሀ) የመንግስት ሰራተኛ ለ) ነጋዴ

ሐ) ገበሬ መ) የጉልበት ሰራተኛ

ሠ) ስራ አጥ ረ) ተማሪ

ሰ. ሌላ ካለ __________________________

ለ. መሰረታዊ ጥያቄዎች

1. ስለ ሳንባ ነቀርሳ በሽታ ያውቃሉ? መልስዎ አዎን ከሆነ ወደ ጥያቄ ቁጥር 2 ይሂዱ

ሀ) አዎን ለ) አላዉቅም

2. በመጀመርያ የሰሙት ከማን ነዉ

ሀ) ከቤተሰብ፣ከጓደኛ፣ከጎረቤት፣ ከስራ ባልደረባ ለ) ከቲቢ በሽታ ታማሚ

ሐ) ከጤና ባለሙያ (እንደ ጤና ኤክሰቴንሽኖችና ነርሶች) መ) ከመምህራን

ሠ) ከሚዲያ (እንደ ራዲዮና ቴሌቭዥን) ረ) ከመጽሓፍ፣ በራሪ ወረቀቶች፣ ፖስተሮችና ሌሎች ህትመቶች ሰ) ሌላ ካለ ____________________________

3. ከዚህ በፊት በሳንባ ነቀርሳ የታመመ ሰዉ አይተዉ ያዉቃሉ? ሀ) አዎን ለ) አላየሁም

4. ለተራ ቁጥር 3 መልስዎ አዎን ከሆነ የት? _________________________

____

5. በሽታዉ ተላላፊ ነዉ? ሀ) አዎን ለ) አይተላለፍም ሐ)አላዉቅም

6. ይህንን በሽታ የሚያስከትለዉ ምንድነዉ?

ሀ) ባክቴርያ/ጀርም ለ) ቀዝቃዛ ዓየር ሐ) የምግብ እጥረት መ) ሲጋራ ማጨስ

ሠ) ጫት መቃም ረ) አቧራ ሰ) አላዉቀዉም

ሸ) ሌላ ካለ _____________________________________

7. ይህ የሳንባ ነቀርሳ በሽታ (ቲቢ) የሚያጠቃዉ

ሀ) ሳንባ ብቻ ለ) ከሳንባ በስተቀር ሌላ የሰዉነት አካል ሐ) ሳንባና ሌላ የሰዉነት አካል

መ) አላዉቅም ሠ) ሌላ ካለ _________________________________

8. ይህ በሽታ ይድናል? ሀ) አዎን ለ) አይድንም ሐ) አላዉቅም

9. ህመም ሲሰማዎት በመጀመርያ ወዴት ይሔዳሉ

ሀ) ወደ ባህላዊ ህክምና ለ)ወደ እምነት ቦታ ሐ)የግል ክልንክ

መ) መንግስታዊ የጤና ተቋማት እንደ ጤና ኬላ፣ ክልንክ፣ ጤና ጣቢያ ወይም ሆስፒታል

ሠ) መንግስታዊ ባልሆኑ የጤና ተቋማት (ኤን ጂ ኦ)

ረ) ሌላ ካለ _____________________________________

10. ከሶስት ሳምንት የበለጠ ሳል ሲያስልዎ ወደ ጤና ተቋም ይሔዳሉ?

ሀ) አዎን ለ) አልሔድም ሐ) አልፎ አልፎ

11. በእርስዎ እይታ በሳንባ ነቀርሳ (ቲቢ) የሚጠቃ ማነዉ

ሀ) የትኛዉም ሰዉ ለ) ድሃ ብቻ

ሐ) ቤት የሌላቸዉ ሰዎች ብቻ መ) ጠጪዎች ብቻ

ሠ) መድሃኒት ተጠቃሚዎች ብቻ ረ) በኤች አይ ቪ/ ኤድስ የተያዙት ሰዎች ብቻ

ሰ) ጫት የሚቅሙት ብቻ

ሸ) ሌላ ካለ ____________________________

12. በዚህ በሽታ የተያዘ ሰዉ እንዴት ልድን ይችላል?

ሀ) ባህላዊ መድሃኒት በመጠቀምለ) መድሃኒት ሳይወስዱ እረፍት በመዉሰድ

ሐ) በመጸለይመ) የበሽታዉን መድሃኒት በታዘዘዉ መሰረት በመዉሰድ

ሠ) አይድንምረ) አላዉቅም

ሠ) ሌላ ካለ __________________________________

13. ከዚህ በፊት በቲቢ በሽታ ታመዉ ያዉቃሉ ሀ) አዎን ለ) አላዉቅም

14. አብዘኛዉን ግዜ በዚህ በሽታ የምታመሙት በየትኛዉ የእድሜ ክልል የሚገኙት ሰዎች ናቸዉ

ሀ) ከወሊድ እስከ 15 አመት የእድሜ ክልል

ለ) ከ15 እስከ 54 አመት (የሰራተኝነት የእድሜ ክልል)

ሐ) ከ54 አመት በላይ

መ) ሁሉም የእድሜ ክልል እኩል ይጠቃሉ

ሠ) አላዉቅም

15. አንድ ሰዉ በሳንባ ነቀርሳ በሽታ እንዴት ልያዝ ይችላል

ሀ) በመጨባበጥ ለ) በአየር አማካኝነት የቲቢ ህመምተኛ ስያስለዉ/ስያስነጥሰዉ

ሐ) አብሮ በሙብላት መ) በደም

ሠ) እቃዎችን በመነካካት (የበር እጀታ፣ የተጓዦች መያዣ በመጨበጥ)

ሰ) በግብረ ስጋ ግንኙነት ረ) አላዉቅም

ሸ) ሌላ ካለ __________________________________

16. በሚያስነጥስዎት ወይም በሚያስልዎት ግዜ አፍዎትንና አፍንጫዎትን ይሸፍናሉ?

ሀ) አዎን ለ) አልሸፍንም ሐ) አልፎ አልፎ

17. ከዚህ በፊት መድሃኒቱን የምለማመድ ቲቢ መኖሩን ሰምተዉ ያዉቃሉ?

ሀ) አዎን ለ) አላውቅም

18. ባክቴርያዉ መዲሃኒቱን እንዲለማመድ የሚያደርገዉ/ጉት ምን ይመስልዎታል (ከአንድ በላይ መምረጥ ይቻላል)

ሀ) የህክምና ባለሙያ ባዘዘዉ መሰረት መድሃኒቱን አለመዉሰድ

ለ) መድሃኒቱ በሚፈለገዉ የባክቴሪያ ሰዉነት ላይ አለመጣበቅ (የባክቴርያዉ መለወጥ)

ሐ) መዲሓኒቱን የተለማመደ ባክቴርያ ከህመምተኛ ወደ ጤነኛዉ ስተላለፍ

መ) አላዉቅም

ሠ) ሌላ ካለ __________________________________

19. መድሃኒቱን የተለማመደ ባክቴርያ ለመዳን የሚወስደዉ ጊዜ

ሀ) መድሃኒቱን ካልተለማመደዉ እኩል ነዉለ) መድሃኒቱን ካልተለማመደዉ ያነሰ ጊዜ ነዉ

ሐ) መድሃኒቱን ካልተለማመደዉ የበለጠ ጊዜ ነዉ

መ) አይድንም ሠ) አላዉቅም

ረ) ሌላ ካለ ___________________________________

20.አንድ ሰዉ ከቲቢ በሽታ እንዴት እራሱን ልጠብቅ ይችላል (ከአንድ በላይ መምረጥ ይቻላል)

ሀ) አለመጨባበጥ

ለ) ሳል ወይም ማስነጠስ ስኖር አፍና አፍንጫን በመሸፈን

ሐ) እቃዎችን በጋራ አለመጠቀም

መ)እጅን በመታጠብ

ሠ) መስኮቶችን በመዝጋት

ረ) በደንብ በመመገብ

ሸ) በመጸለይ

21. ጫት ይቅማሉ? መልስዎ አዎን ከሆነ ወደ ጥያቄ ቁጥር 22 ይሂዱ

ሀ) አዎን ለ)አልቅምም

22. በአማካይ በሳምንት ምን ያህል ጊዜ? _________________

23. ጫትን የሚቅሙ ከሆነ ከማን ጋር ነዉ

ሀ) ከጓደኞቼ ጋር ለ) ለብቻዬ ሐ) አልፎ አልፎ ከጓደኞቼ ጋር መ. ሌላ ካለ

24. በተጣበበ ቤት ዉስጥ መስኮት ዘግቶ ጫት መቃም የበለጠ ለቲቢ በሽታ ያጋልጣል፡፡

ሀ) እዉነት ለ) ሀሰት ሐ)አላዉቅም

25. ከቲቢ ጋር ቁርኝት ያላቸዉን በሽታዎች ዘርዝር

____

26. የቲቢ በሽታ ከሰዉ ወደ እንስሳት ከእንስሳት ወደ ሰዉ ይተላለፋል?

ሀ) አዎን ለ)አይተላለፍም ሐ) አላዉቅም

27. አዎን ከሆነ እንዴት ____

28. የሳንባ ነቀርሳን በሽታ ምልክቶች ይዘርዝሩ

____

____

29. በቲቢ በሽታ የታመመን ሰዉ በቤት ውስጥ እንዴት ይንከባከባሉ? እባክዎትን ያብራሩ፡፡

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Guyyaa ______________

Odeeffannoo Dabalataa III. Gaafilee

Pirojaktiin qorannoo PhD kanaa faffacaa'ina dhibee saanbaa fi wantoota kanaan wal qabatan naannoo Amaaraa Godina addaa Oromiyaa fikibba Walloo, kaaba baha Itiyoophiyaa baruuf. Baakiteeriiyyaan kun qorichaan walbaruu issaatifii beekumsa, ilaalcha fi hojjii irra olchuu yaada dhibee kanaaf qabdan ilaaludha. Dabalataaniis wal-qunamtii TBn helmentii (TB-helminth co-infection) wajjin qabu ni ilaalama. Qorannoon dhibee kanaa caasaa dhibicha naanno kanatti ofirraa ittisaniif kaayoo gudaa qaba.Pirojaktichi Yunivarsiitii finfinee maayikirobiyaal, selulari fi molokiyular baayoolojji dipartimantii irra beekumsa argatee jira. Projaktiin kun naannoo Amaaraa godina oromiyaa, anaa kanaa fi bufata fayaa kana irraa eyyama argatee jira. Deebin Gaafilee armaan gadii kanaaf gootan qorannoo kanaaf ga'e guddaa qaba.

waraqaa gaaffii kana irratti maqaa keessan bareessun hin barbaachisu. Xumurarrattis, yeroo keessan kenitanii gaafilee kana deebisuuf sochii gootan hundaafu baayyee issin galateeffana.

Note: ☞ Malatoo tana ✓ saanduqa sirrii ta'e keessatti godhaa

☞ Gaafilee filanno qabaniif deebii keessanitti mari

B. Odeeffano Waligalaa

1.Koodin hirmaataa/ttu _________________ Naanno Amaaraa Godina ____________________

Aanaa ______________________________ Ganda ____________________________

2. Umurii ___________________

3. Saala Dhiira ☐ Nadheen/Dubree ☐

4. Haala gaa'elaa kan hin fuune/heerumne ☐ kan fudhe/heerumte ☐ kan hiike/hiikte ☐

5. Baay'inn maatii ______________

6. Saba ________________

7. Amantaa _________________

8. Ga'umsi barumsaa

a) Hin baranne ☐ b) Sadarkaa tokkoffa ☐ c) Sadarkaa lamaffaa ☐

d) Barumsa olaanaa – sertefketti ☐ Dipilomaa ☐ Digirii ☐ Maasteri fi isaa ol ☐

9. Wa'e hojii

a) Hojetaa motummaa b) Daldalaa

c) Qonnaan bulaa d) Hojetaa humnaa

e) Hojii hinqabu f) Barataa

g) kan hanbiraa yoo jiraate ____

B. Gaafilee Bu'uraa

1. Waa'e dhibee sombaa beektu? Deebin keessan "eyyen" yoo ta'e gara lak. 2tti derbaa

a) Eyyen

b) Hinbeeku

2. Yeroo duraaf enyurraa dhageessan?
   a) Maatii, hiriyaa, olaa fi miiltoo hojii
   b) Nama dhibee sombaa dhibame irraa
   c) Hojjatoota fayyaa irraa (kan akka hojjatoota eksitenshinii fayyaa fi narsoota)
   d) Barsiftotarraa
   e) Miidiyaa (kan akka raadiyoo fi TV)
   f) Kitaaba, bareeffama postarii fi kan adda addaa
   g) Kan biraa yoo jiraate mee barressi ____

3. Kana dura nama dhibee sombaattin (TB) dhibaban argitanii beektu?
   a) Eyyen   b) Hin argine

4. Gaafii 3ffaa deebin keessan "eyyen" yoo ta'e essatti?
____

5. Dhibeen kun namarraa namatti ni daddarbaa?
   a) Eyyen   b) Hin derbu   c) Hin beeknu

6. Dhibee TB kan wanti fidu maali dha?
   a) Baakiteeriyaa/jarmi   b) Qilleensa qoru   c) Hir'ina nyaataa   d) Tamboo xuxu
   e) Caattii nyaachuu   f) Hawwaara   g) Hin beeku
   h) Kan biraa yoo jiraate barressaa ____

7. Baakteeriyaan TB kan miidhu
   a) Somba qofa   b) Sombaan ala qaama biraa
   c) Sombaa fi qaama biraas   d) Hin beeku
   e) Kan biraa yoo jiraate barressi ____

8. TBn dhibee fayyu?
   a) Eyyen   b) Moti
   c) Hin beeku

9. Yennaa dhukubffatu jalqabarratti garam deemta?
   a) Gara qoricha habashaa   b) Gara mana amantaa   c) Kilinika dhuunfaa
   d) Gara mana fayyaa motummaa kan akka kilinikaa, bufata fayyaa fi hospitaalaa
   e) Kilinikoota mottumaan ala socho'an (NGO)
   f) Kan biraa yoo jiraate barressi ____

10. Yerroo dheeraf yoo si'uke (siqufaasise) gara mana yaalaa dhaqxanii ni ilaalamtaa (torban 3 ol)?   a) Eyyen   b) Hin dhaqinu   c) Darbee darbee

11. Ilaalcha keessanitti dhukuba TBttin eenyufaatu dhibamu danda'a?
   a) Abbuma fe'e   b) Namoota qabeenyaa hinqabne (iyyessa) qofa
   c) Namoota mana hinqabne qofa   d) Namoota dhugaati dhugan qofa
   e) Namoota qoricha fayyadaman qofa   f) Namoota HIV/AIDS qaban qofa
   g) Namoota caatii nyaatan qofa
   h) Kan biraa yoo jiraate barressi ____

12. Namni dhibee TB qabu akkamitti fayyuu danda'a?
   a) Qoricha habashaa fayyadamuun
   b) Qoricha malee boqonaa fudhachuun
   c) Uumaa kadhaadhan
   d) Qoricha ajajame seeran fudhachun
   e) Hin fayu
   f) Hin beeku
   g) Kan biraa yoo jiraate barressi ____

13. Kanaan dura dhibee TBttin dhibamtani beektaa?
   a) Eyyen
   b) Hin beeknu

14. Baayyinaan TBn nama umurii kamii qabuu danda'a?
   a) Dhalootaa hanga Waggaa kudhashanii _______________________________________
   b) Waggaa kudha shanii hanga oggaa shantamii afurii (umurii hojii) ________________
   c) Waggaa shantamii afurii ol ____
   d) Umurii hundi walqixa ____
   e) Hin beeku ____

15. TBn akkammit nama qabu danda'a?
   a) Harka wal-qabachuun
   b) Namni TB qabu yennaa qufa'u/hukkamu qillensa irraan
   c) Iddoo tokkotti nyaachun
   d) Dhiigaan
   e) Iddoo ummatin jiruti mi'a tutuqun (kan akka qabanaa balbalaa, qabanaa geejjiba keessaa fi kkf)
   f) Walqunnantii saalattiin
   g) Hin beeku
   h) Kan biraa yoo jiraate barressaa ____

16. oggaa qufaatu fi namni biraa sibiraa qufa'u affaan kee ni haagugatu/qabattu
   a) Eyyen
   b) Moti
   c) Darbee darbee

17. Baakteeriyaan dhibee TB fidu qorricha baree akka ofirraa ittisu dhageesee beektaa?
   a) Eyyen   b) Hin dhageenye

18. Baakteeriyich akkamitti qorricha baru danda'a (tokko ol filachuun ni dana'ama)
   a) Akka ogeessi fayyaa ajajetti qoricha fudhachuu dhiisuun
   b) Haalli uumama baacteeriyichaa jijiramuu isaattin
   c) Baakteeriyaan qoricha bare dhibamaa tokko irra nama biraatti darbuun
   d) Nama TB qabu hunda irraa namatti darbuun
   e) Hin beeku
   f) Kan biraa yoo jiraate mee Barresi ____

19. Baakteeriyaan qoricha bare fayyuf hangam fudhata

a) Issaan qoricha hin barren wal-qixa

b) Issaan qoricha hin barren gadi

c) Issaan qoricha hin barren ol

d) Hhin fayyu

e) Hin beeku

20. Namni tokko TB irraa akkamitti of ittisu danda'a (Tokko ol filachuu ni dandeessa)

a) Harka wal qabachuu dhiisun b) Oggaa qufa'an affaan fi funyaan qabachuun

c) Nyaata wajjin nyaachu dhiissun

d) Mi'a namooni biraa tuqan tuquu dhiissun

e) Foddaa manaa cufudhan

f) Nyaata gaarii nyaachun

g) Kadhachuun

h) Hin beeku

i) Kan biraa yoo jiraate mee barressi ____

21. Caatii ni qaamaatu/nyaattu? Yoo ta'e gara lak. 22

a) Eyyen b) Moti

22. walumaa galatti Torbanitti yeroo meeqa? _______________________________

23. Yeroo caati qamaatan/nyaatan

a) Hiriyyaa wajjin b) Kobaa

c) Yeroo tokko tokko hiriyyaa wajjin

d) Kan biraa yoo jiraate mee barressi ____

24. Mana foddaan isaa cuffame keessati tutaniin caatii qama'un irra jirreessatti dadarbu TBtiif nama saaxila

a) Dhugaa b) Soba c) Hin beeku

25. Dhibee biraa TB dhaan kan wal qabatan barreesi

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26. Dhibeen TB nama irraa gara beeladaatti, beelada irraa immoo gara namaati ni darbaa

a) Eyyen b) Moti c) Hin beeku

27. Deebin kee eyyen yoo ta'e, akkamitti ____

28. Mallatowwan TB barressi?

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29. Nama TB qabu mana keessatti akkamitti kununsitu? Mee sirriti ibsaa

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Appendix IV. Study Protocols

A. Ziehl–Neelsen acid-fast staining procedures

1. Dropping of the cells in suspension onto a slide
2. Air drying the liquid and heat fixing of the cells
3. Flooding the slide with Carbol Fuchsin to stains every cell
4. Heating to dry and rinsing in tap water
5. Flooding with a mild solution of hydrochloric acid in isopropyl alcohol to de-stain the Carbol Fuchsin. Only those of non-acid-fast bacteria will be counter stained by the alcohol due to lack of thick, waxy lipid layer unlike those of acid-fast bacteria.
6. The stains (Carbol Fuchsin) will be removed from cells that are unprotected by a waxy lipid layer
7. Counter staining of cells with methylene blue and viewing on a microscope under oil immersion makes the non-acid-fast bacteria as blue color while the acid-fast bacteria as maintaining the original red color of carbol fuchsin.

B. Direct stool test

1. A drop of saline (0.9%) was placed in the middle of microscopic slide
2. A small proportion (about size of a match stick head) of the specimen was picked up and mixed with the drop of saline.
3. The mixed drop of saline was coverd with cover slip
4. The mounted slide was put on the microscopic stage and focused with low power objective.
5. The entire cover slip area was systematically observed and when organisms or suspicious materialwas seen switched to dry high power objective (40x) to observe the detailed morphology.

C. Kato-Katz

1. A screening meshwas put over the sizeable stool specimen on the plastic sheet
2. The stool under the screen was scrapped with spatula so that the stool was sieved out of the screen
3. The sieved stool with spatula was collected and filled in the holes of templates on the microscopic slides (Each hole of the template form 41.7mg stool sample).
4. The template was carefully removed so that cylinder of feces was left on the slide.

5. The slide with stool was covered by pre-soaked cellophane strip (with Glycerol-malachite green solution).

6. Gently slided over the sample with another slide so that the stool was spreaded evenly.

7. The prepared slide was left for at least 24 hrs to clear the smear.

8. The slide was examined using a microscope in a systematic manner so that the number of eggs for each species was recorded.

9. Finally, number of eggs was multiplied by appropriate number to give number of eggs per gram of faeces (see Inlet-information of the Kato-set)

D. SAF (Sodium acetate-acetic acid-formalin solution)

1. Stool sample with a size of match head (approximately 1g) was placed into a centrifuge tube containing 10ml of SAF and mixed with a wooden stick thoroughly
2. The tube was closed and agitated vigorously
3. The stool solution was filtered using a funnel with gauze
4. The filtered sample was centrifuged for 1 minutes at 2000rpm
5. The supernatant was decanted or removed
6. 7ml of saline was added and mixed with a wooden stick
7. 3ml of ether was added on it
8. The tubes were closed with a rubber stopper and shaked well keeping the thumb on the stopper
9. The rubber stopper was removed carefully (pressure!) and the tube is centrifuged for 5 minute at 2000 rpm.
10. Four layers have been detected after centrifugation as ether, detritus, saline and sediment from top to bottom
11. The first three layers were removed and only the sediment was left
12. If the sediment was greater than 1ml the concentration was repeated with saline or ether.
13. Finally, the sediment was mixed and its drop was placed on microscopic slides and covered with a cover slip for microscopic examination.
14. Helminthic eggs were searched with 10X objective followed by protozoan parasites using an objective of 50X with oil immersion.

E. Preparation of the freezing media

1. Preparation of 7H9 media

- For 450ml of distilled water, 2.35gm of 7H9 powder was added
- 1ml of glycerol was added and mixed
- Autoclaved at 121 ^{0}C for 10 minutes
- The solution was let until its temperature comes to 45 ^{0}C
- 50ml of middle brook ADC enrichment (OADC) was added

2. Preparation of freezing media

- 50ml of the prepared 7H9 media was taken and 25ml of glycerol was added to it
- 25ml of distilled water was added and mixed well
- Finally, the solution was filtered into sterile container using 0.22µm filter and remained in use for 3 weeks

3. Freezing the *Mycobacterium* colony

- 2-3 colonies of the *Mycobacterium* colonies from the LJ media was taken and added to a 1.8ml nunc tube containing 1ml of the freezing media and mixed thoroughly

The freezed colonies in a nunc tube were preserved at -80 ^{0}C deep freezer

Appendix V: Sequences of the spacer specific oligonucleotides on the membrane of spoligotyping

1:ATAGAGGGTCGCCGG**T**TCTGGATCA 1)

2: CCTCATAATTGGGCGACAGCTTTTG 2)

3: CCGTGCTTCCAGTGATCGCCTTCTA

4: ACGTCATACGCCGACCAATCATCAG

5: TTTTCTGACCACTTGTGCGGGATTA

6: CGTCGTCATTTCCGGCTTCAATTTC

7: GAGGAGAGCGAGTACTCGGGGCTGC

8: CGTGAAACCGCCCCCAGCCTCGCCG

9: ACTCGGAATCCCATGTGCTGACAGC

10: TCGACACCCGCTCTAGTTGACTTCC

11: GTGAGCAACGGCGGCGGCAACCTGG

12: ATATCTGCTGCCCGCCCGGGGAGAT

13: GACCATCATTGCCATTCCCTCTCCC

14: GGTGTGATGCGGATGGTCGGCTCGG

15: CTTGAATAACGCGCAGTGAATTTCG

16: CGAGTTCCCGTCAGCGTCGTAAATC

17: GCGCCGGCCCGCGCGGATGACTCCG

18: CATGGACCCGGGCGAGCTGCAGATG

19: TAACTGGCTTGGCGCTGATCCTGGT

20: TTGACCTCGCCAGGAGAGAAGATCA

21: TCGATGTCGATGTCCCAATCGTCGA

22: ACCGCAGACGGCACGATTGAGACAA

23:AGCATCGCTGATGCGGTCCAGCTCG

24: CCGCCTGCTGGGTGAGACGTGCTCG

25: GATCAGCGACCACCGCACCCTGTCA

26: CTTCAGCACCACCATCATCCGGCGC

27: GGATTCGTGATCTCTTCCCGCGGAT

28: TGCCCCGGCGTTTAGCGATCACAAC

29: AAATACAGGCTCCACGACACGACCA

30: GGTTGCCCCGCGCCCTTTTCCAGCC

31: TCAGACAGGTTCGCGTCGATCAAGT

32: GACCAAATAGGTATCGGCGTGTTCA

33: GACATGACGGCGGTGCCGCACTTGA

34: AAGTCACCTCGCCCACACCGTCGAA

35: TCCGTACGCTCGAAACGCTTCCAAC

36: CGAAATCCAGCACCACATCCGCAGC

37: CGCGAACTCGTCCACAGTCCCCCTT

38: CGTGGATGGCGGATGCGTTGTGCGC

39: GACGATGGCCAGTAAATCGGCGTGG

40: CGCCATCTGTGCCTCATACAGGTCC

41: GGAGCTTTCCGGCTTCTATCAGGTA

42: ATGGTGGGACATGGACGAGCGCGAC

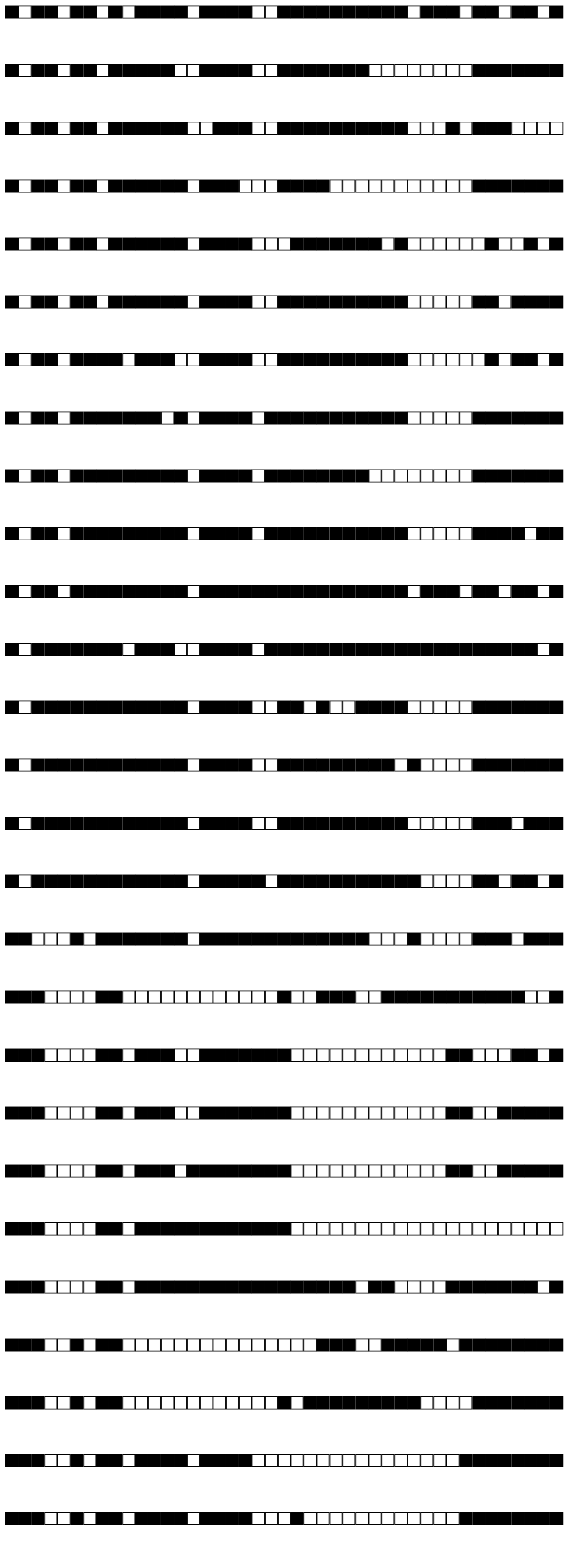
43: CGCAGAATCGCACCGGGTGCGGGAG

Appendix VI. Locus designations and PCR primer sequences used in this study for the 24-locus MIRU-VNTR set

Multiplex	Locus	Alias	Repeat unit length (bp)	PCR primers (5' to 3')
Mix 1	580	MIRU 4	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC
	2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
	802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
Mix 2	960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
	1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
	3192	MIRU 31	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
Mix 3	424	42	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC
	577	43	58	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACGCGCAAATTGTGA
	2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
Mix 4	2401	47	58	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA
	3690	52	58	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
	4156	53	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
Mix 5	2163b	QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
	1955		57	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
	4052	QUB-26	111	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCGAT
Mix 6	154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT
	2531	MIRU 23	53	CTGTCGATGGCCGCAACAAAACG AGCTCAACGGGTTCGCCCTTTTGTC
	4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT
Mix 7	2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTACTTGTA
	2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
	3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA
Mix 8	2347	46	57	GCCAGCCGCCGTGCATAAACCT AGCCACCCGGTGTGCCTTGTATGAC
	2461	48	57	ATGGCCACCCGATACCGCTTCAGT CGACGGGCCATCTTGGATCAGCTAC
	3171	49	54	GGTGCGCACCTGCTCCAGATAA GGCTCTCATTGCTGGAGGGTTGTAC

Appendix VII. Description of spoligotype patterns, lineages and shared-types of 104 valid *M. tuberculosis* isolates collected from northeast Ethiopia, April 2015 to January 2017

Webdings format	Octal code	Family	Major Lineage	Sub-lineage	SIT	No*
□□■■□■■□■□■■■□□■□□□□□□□■■■■■■■■□□□□□■■■■□□□	155344037740740	H37Rv	EUA	T-Tuscany	Orphan	1
■□□□□□□□■■■■■■□■■□■□■■■■■■□■□□□■□□□□■■■□■■■	401766576420731	Haarlem3	EUA	H4-Ural-2	Orphan	1
■□■□□■□□□□□□□□□□□□□□□□□■■■■■■■□□□□□■■■■■■■	510000017740771	T3	EUA	T1-RUS2	Orphan	1
■□■□□■□□■□□□□□□■□□□□□□□■■■■■□■■□□□□□□□■□■■□■	511004037300261	H37Rv	EUA	H	Orphan	1
■□■□□■□□■□■■■□□■■□□□□□□□□□□□□□□□□□□□■■■■■■■	511346000000771	LAM8	EAI	CAS	Orphan	1
■□■□□■□□■■■■■■□■■■■□■■□□□□□□□□□□□□□■■□□■■■■■	511767540003171	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■□□■■■■■■□■■■■□■■□□□□□□□□□□□□□■■■■■■■■■	511767540003771	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■□■■□■■■■□■■■■□□□□□□□□□□□□□□□□■■■■■■■	513367400001771	CAS	EAI	CAS1-Kili	Orphan	1
■□■□□■□■■□■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■	513377400001771	CAS	EAI	CAS1-Kili	Orphan	2
■□■□□■□■■■■■■■□■■■■□■■□□□□□□□□□□□□□■■■□■■■■	513767540003571	CAS	EAI	CAS1-Delhi	Orphan	1
■□■□□■■□■□□□□□□■□□□□□□□■■■■■□■■□□□□□□□■□■■□■	515004037300261	H37Rv	EUA	H	Orphan	1
■□■□□■■□■□■□□□□■■■□□□□■■■■■■■■■■□□□□□□■□■■□■	515207037740261	H37Rv	EUA	T	Orphan	2
■□■□□■■□■□■□□□□■■■□■□□■■■■■■■■■■□□□□□□■□■■□■	515207237740261	T1	EUA	T	Orphan	1
■□■□□■■□■□■■■□□■■□■□□□■■■■■■■■■■□□□□□□■■■■□■	515346437740361	H37Rv	EUA	T	Orphan	1
■□■□□■■□■□■■■□□■■■□■■□■■■■■■■■■■□□□■□□■□■■□■	515347337742261	Family 33	EUA	T4	Orphan	1
■□■□□■■□■□■■■□□■■■□■■■■■■■■■■■■■□□□■□□■□■■□■	515347377742261	Family 33	EUA	T4	Orphan	2
■□■□□■■□■□■■■□□■■■■■■□■■■■■■■■■■□□□■□■■□■■□■	515347737742661	Family 33	EUA	Manu2	Orphan	1
■□■□□■■□■□■■■□□■■■■■■■■■■■■■■■■■□□□■□□■□■■□■	515347777742261	Family 33	EUA	Manu2	Orphan	2
■□■□□■■□■□■■■□■■■■■■■■■■■■■■■■■■■■□□■□■■□■■■■	515357777762671	Family 33	EUA	Manu2	Orphan	1
■□■□□■■■■□■■■□□■■■■□□□□□■□□■■■■□□□□□□□■□■■■■	517347404740271	LAM9	EUA	Turkey	Orphan	1
■□■□□■■■■□■■■□□■■■■□□■■■■■■■■■■■□■□■□■■□■■□■	517347477752661	Family33	IO	Manu1	Orphan	1
■□■■□■■□■□■□□□□■■■□■□□■■■■■■■■■■□□□□□□□■□■■□■	555207237700261	Haarlem3	EUA	T	Orphan	1
■□■■□■■□■□■■■□□■■■□■□□■■■■■■■■■■□□□■□■■□■■□■	555347237742661	Family33	EUA	Manu2	Orphan	1
■□■■□■■□■□■■■□□■■■■■□□■■■■■■■■■■□□□■□■■□■■□■	555347637742661	Family 33	EUA	Manu2	Orphan	1
■□■■□■■□■□■■■■■□■■■■□□□■■■■■■■■□□□□□□□■□■■□■	555367437600261	H37Rv	EUA	T	Orphan	1



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	555767074000771	Haarlem1	IO	H1	Orphan	1
	555767437640221	H37Rv	EUA	T	Orphan	1
	555767477740671	H37Rv	EUA	T	Orphan	1
	557347477740261	H37Rv	EUA	T	Orphan	1
	557727577740771	T1	EUA	T	Orphan	1
	557767577400771	Haarlem3	IO	H4-Ural-2	Orphan	1
	557767577740751	T1	EUA	T	Orphan	1
	557767777756661	Family33	IO	Manu1	Orphan	1
	577347577777761	Family33	IO	Manu1	Orphan	1
	577767464740771	H37Rv	EUA	T	Orphan	1
	577767477720771	H37Rv	EUA	H3	Orphan	1
	577767477740731	H37Rv	EUA	H37Rv	Orphan	1
	577767677760661	T1	EUA	T	Orphan	1
	613767777420731	Haarlem3	EUA	H3-Ural-1	Orphan	1
	703000047177741	Family33	EAI	Manu_ancestor	Orphan	1
	703347740003061	CAS	EAI	CAS1-Delhi	Orphan	1
	703347740003171	CAS	EAI	CAS1-Delhi	Orphan	1
	703357740003171	CAS	EAI	CAS1-Delhi	Orphan	1
	703377740000000	Family34	EAI	CAS	Orphan	1
	703377777303761	Family33	IO	Manu2	Orphan	1
	713000007175771	Family33	IO	H	Orphan	1
	713000057760771	T3	EUA	T3-ETH	Orphan	1
	713367400001771	CAS	EAI	CAS1-Kili	Orphan	1
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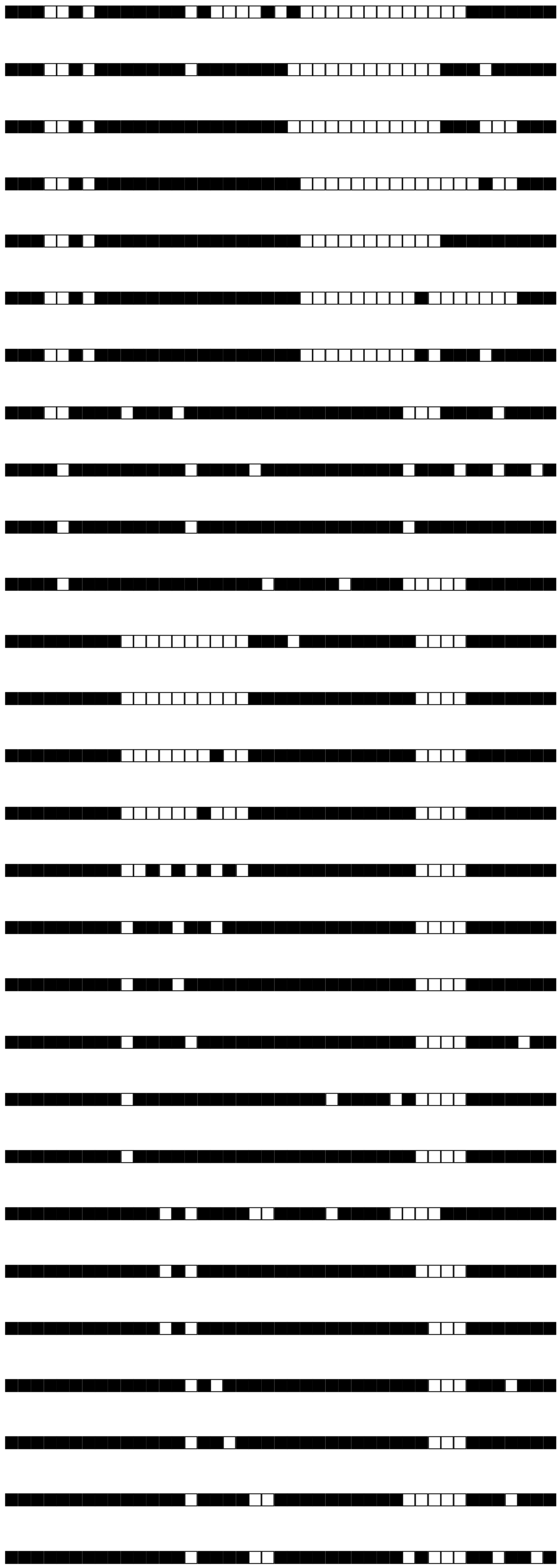

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713777760000231	Haarlem1	EUA	H1	Orphan	1
713777760003771	EAI4	IO	CAS1-Delhi	Orphan	1
713777760010031	Haarlem1	IO	H1	Orphan	1
713777760013571	EAI4	IO	CAS1-Delhi	Orphan	2
717357777743671	Family 33	EUA	Manu2	Orphan	1
757767577756661	Family33	IO	Manu1	Orphan	1
757767777757771	Family33	IO	Manu1	Orphan	1
757777676740771	T1	EUA	T	Orphan	1
777000357760771	T3	EUA	T3-ETH	Orphan	1
777000377760771	T3	EUA	T3-ETH	149	3
777002377760771	T3	EUA	T3-ETH	Orphan	1
777004377760771	X1	EUA	T3-ETH	Orphan	1
777125377760771	T1	EUA	T	Orphan	1
777355777760771	T1	EUA	T	Orphan	1
777357777760771	T1	EUA	T	1475	1
777367777760751	T1	EUA	T	Orphan	1
777377775720771	Haarlem3	EUA	H3	Orphan	1
777377777760771	T1	EUA	T	1166	1
777727475703771	Family33	IO	Manu2	Orphan	1
777727777760771	T1	EUA	T3	1547	1
777727777770771	T1	EUA	Manu3	Orphan	2
777765777770731	T1	EUA	Manu3	Orphan	1
777766777770771	T1	EUA	Manu3	Orphan	1
777767477740731	H37Rv	EUA	T	Orphan	1
777767477750661	Family33	EUA	H37Rv	Orphan	1

	777767577776761	Family33	IO	Manu3	Orphan	1
	777767777413731	EAI5	IO	EAI1-SOM	1251	1
	777767777660771	T1	EUA	T	Orphan	1
	777767777720631	Haarlem3	EUA	H3	1802	1
	777767777730771	T1	EUA	Manu3	Orphan	1
	777767777770700	T1	EUA	Manu3	Orphan	1
	777767777770731	T1	EUA	Manu3	Orphan	1
	777767777770771	T1	EUA	Manu3	Orphan	1
	777777774020771	Haarlem1	EUA	H1	47	1
	777777777760751	T1	EUA	T	612	1
	777777777760771	T1	EUA	T	53	2
	777777777770771	T1	EUA	Manu3	1378	2

key: CAS = Central Asian; EAI = East-African India; EUA = Euro-American ; H = Haaralem; IO = Indo-Oceanic;LAM = Latin American Mediterranean and No* = number of isolates with similar pattern.

Appendix VIII. Dissemination of the study findings

A. Published paper and manuscripts prepared for publication

1. **Gashaw, F.,** Bekele, S., Mekonnen, Y., Medhin, G., Ameni G. and Erko, B. (2019). High helminthic co-infection in tuberculosis patients with undernutritional status in northeastern Ethiopia.*BMC Infect. Dis. Poverty*.**8**:88.
2. **Gashaw, F.,** Yenew, B., Amare, M., Mekonnen, Y., Erko, Medhin, G., B., Gumi, B. and Ameni, G. (20xx).Phenotypic and Genotypic Drug Sensitivity Patterns of *Mycobacterium tuberculosis* in Oromia Special Zone and South Wollo Zone, Northeastern Ethiopia. (Manuscript Prepared)
3. **Gashaw, F.,** Zewde, A., Wondale, B., Mekonnen, Y., Medhin, G., Erko, B., Gumi, B. and Ameni, G.(20xx). Molecular characterization of *Mycobacterium tuberculosis* in Oromia Special Zone and South Wollo Zone, Northeastern Ethiopia. (Manuscript under preparation)
4. **Gashaw, F.,** Tedla, E., Erko, B., Ameni, G. and Mekonnen, Y., (20xx). Knowledge, attitude and preventive practice towards tuberculosis among tuberculosis cases visiting the health facilities in Northeastern Ethiopia, Amhara Regional State. (Manuscript under preparation)

B. Presentations on International and National Conferences

1. Molecular Epidemiology, Drug Sensitivity Patterns and Parasitic Co-infection of *Mycobacterium tuberculosis* in Amhara Regional State, Northeastern Ethiopia (International Conference Prepared by Kotebe Metropolitan University in Ethiopia, August, 2019).
2. Molecular Epidemiology, Drug Sensitivity Patterns and Parasitic co-infections of *Mycobacterium tuberculosis* in Amhara Regional State, Northeastern Ethiopia. (International, H3 Africa consortium in Botswana, May 11-15, 2017).
3. Tuberculosis and parasitosis co-morbidities in northeastern Ethiopia (National, the13th annual TB research conference in Ethiopia and got a national award with certificate of recognition from Ethiopian Ministry of Health, April, 2018).
4. Prevalence of intestinal helminth infection among school children in Maksegnit and Enfranz towns, Northwestern Ethiopia: with emphasis on *Schistosoma mansoni* infection. (The 1st National Neglected Tropical Diseases (NTDs) Research Conference Jigjiga, 22-23 August 2016).

DECLARATION

I, the undersigned, declare that this PhD dissertation is my own original work and has notbeenpresented for a degree in any other university, and all sources of materials used forthedissertation have been duly acknowledged.

Student: Fikru Gashaw

Signature: _______________

Date: July 2020

Institution: Addis Ababa University

Address: Addis Ababa, Ethiopia