ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Molecular characterization and drug resistance pattern of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia

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A research thesis submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, in partial fulfillment of Master of Sciences Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

June 2020

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Getu Diriba, entitled: "**Molecular characterization and drug resistance pattern of *Mycobacterium tuberculosis* complex among confirmed extra pulmonary tuberculosis cases in Addis Ababa, Ethiopia**" submitted in partial fulfillment of the requirements for the degree of Master of Science in Clinical Laboratory Scinces (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Acknowledgment

I would like to express my deepest gratitude to my advisors Mr. Kassu Desta, Mr. Dessie Abera, Mr. Abebaw Kebede, and Dr. Habteyes Hailu for their guidance during the implementation of the study your scientific advice, support and encouragement gave me the confidence to think critically and work independently to improving the quality of this thesis. I also acknowledge Addis Ababa University, College of Health Sciences, Department of Medical Laboratory Sciences for financial support, and Ethiopian Public Health Institute (EPHI) National Tuberculosis Reference Laboratory (NTRL) for their willingness to support this study by providing laboratory chemicals, reagents, and permission to use laboratory facilities. I would like to express my gratitude to Mr. Bazezew Yenew, Mr. Gebeyehu Asefa, and Mrs. Muluwork Getahun for their boundless support during drug sensitivity testing and spoligotyping analysis. I would also extend my gratitude to Mr. Zemedu Mohhamed for his support on data analysis. I am also thankful to all staff members of EPHI NTRL for their support and fun during the laboratory work of this thesis project.

Lastly, but not least, I would like to acknowledge colleagues in the graduate students of Addis Ababa University, Department of Medical Laboratory Sciences for their support. I would express my gratitude to my family, my wife and all friends who have contributed directly or indirectly to this work and encouraged me.

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List of Abbreviations

AAU	Addis Ababa University
AFB	Acid-fast bacilli
AMK	Amikacin
BCG	Bacille Calmette–Guerin
CAP	Capreomycin
CAS	Central Asia Strain
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
EA	Euro-American
EAI	East-African Indian
EMB	Ethambutol
EPHI	Ethiopian Public Health Institute
EPTB	Extrapulmonary Tuberculosis
ETH	Ethionamide
FNAC	Fine Needle Aspirate Cytology
FQs	Fluoroquinolones
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IO	Indio Oceanic
KAN	Kanamycin
LAM	Latin-American Mediterranean
LAMP	Loop-mediated isothermal amplification
LPA	Line Probe Assay
LTBI	Latent Pulmonary Tuberculosis Infection
MDR-TB	Multidrug Resistance Tuberculosis
MGIT	Mycobacterium Growth Indicator Tube
MOX	Moxifloxacin
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MUT	Mutant

NAA	Nucleic Acid Amplification
NAAT	Nucleic Acid Amplification Test
NTM	Nontuberculous *Mycobacteria*
NTRL	National Tuberculosis Reference Laboratory
OFX	Ofloxacin
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
RIF	Rifampicin
RR	Rifampicin Resistance
SLD	Second-line Drugs
SIT	Spoligotype International Type
SIRE	Streptomycin Isoniazid Rifampicin Ethambutol
SPSS	Statistical Package for Social Scientist
SSPE	Saline-Sodium Phosphate EDTA
STM	Streptomycin
TB	Tuberculosis
VIO	Viomycin
WHO	World Health Organization
WT	Wild type
ZN	Ziehl-Neelsen

Abstract

Background: Molecular characterization and drug-resistant of *Mycobacterium tuberculosis* is a valuable tool in understanding the pathogenesis, diagnosis treatment, and prevention of tuberculosis. However, there is limited information on molecular characterization, and drug-resistant patterns of *Mycobacterium tuberculosis* in patients with extrapulmonary tuberculosis in Ethiopia.

Objective: This study aimed to determine the molecular characteristic and drug resistance patterns of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia

Methods: A laboratory-based cross-sectional study was conducted among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia in a period between November 2019 and April 2020. Drug susceptibility test was performed using BACTEC-MGIT 960 and Line probe assay. Strain identification was performed using the Geno-Type MTBC and spoligotyping technique. Data were entered into the international spoligotyping database to assess the spoligotype patterns of *Mycobacterium tuberculosis*. Data was analyzed by SPSS version 23 and participants' characteristics were presented by number and proportion.

Results: Of 151 *Mycobacterium tuberculosis* complex isolates, 29 (19.2%) were resistant to at least one drug, and 14 (9.3%) were identified as multidrug-resistant tuberculosis. Besides, 21.4% (3/14) of isolates had pre-extensively drug-resistant tuberculosis. S315Tl (11.9% (18/151) was the most common isoniazid mutation observed in the katG gene. The spoligotyping of the 151 isolates resulted in 41 different spoligotype patterns. The most abundantly represented SITs were: SIT149 21.2% (33/151), SIT53 14.6% (22/151) and SIT26 9.6% (14/151). The most predominant lineage was T family 55.0% (83/151), Central Asia Strain 19.2% (29/151) and Haarlem consisted of 8.0% (12/151). Also, 5 (3.3%) isolates had no matching with the database.

Conclusion: The present study showed a high proportion of multidrug-resistant tuberculosis and pre-extensively drug-resistant tuberculosis among EPTB patients. The strain was mostly grouped into SIT149, SIT53, and SIT26. A large scale is required to define the molecular characteristics and drug resistance of *Mycobacterium tuberculosis* in extrapulmonary tuberculosis patients.

Keywords: Extrapulmonary Tuberculosis, Molecular typing, Mutations, multidrug-resistant tuberculosis and pre-extensively drug-resistant tuberculosis

1. Introduction

1.1. Background

Tuberculosis (TB) continues to be an important public health problem mainly in developing countries [1, 2]. Evidence indicated that one-third of the world's population has been infected by *Mycobacterium tuberculosis* (MTB) [3]. The world health organization (WHO) report is also indicated 10 million estimated new TB cases occurred worldwide in 2018; of which 10% of people were living with Human Immunodeficiency Virus (HIV) and about 74% reside in Africa [3]. Five countries: India, Indonesia, China, Philippines, and Pakistan are among the top ten countries in terms of death due to TB worldwide [2]. TB is found in every nation, but the majority of TB cases are concentrated in developing countries [3].

There are two types of TB based on the anatomical sites where the disease manifested. These are pulmonary TB (PTB) and Extra-pulmonary Tuberculosis (EPTB) [4, 5, 6, 7]. EPTB occurs in any part of the body except for the lungs. It becomes an important clinical problem because it accounts for about 15%–20% of TB burden [3]. The prevalence of EPTB is higher in patients co-infected with HIV, especially in endemic countries, though it is less infective than pulmonary tuberculosis [8, 9].

Various biological methods are employed to diagnose EPTB. These are smear microscopy, culture identification, histopathology, tuberculin skin test, serological assays, interferon-gamma release assays and nucleic, acid amplification (NAA) tests [10]. Smear microscopy is widely used in the diagnosis of EPTB but has drawbacks owing to low and variable sensitivity values and could not differentiate between MTB and non-tuberculous mycobacteria (NTM) [11, 12]. Culture identification for MTB also has variable sensitivities in different extrapulmonary specimens [13] with the turnaround time of 4 to 8 weeks and requires skillful technicians [14]. The diagnosis of EPTB from tissue samples is usually made by histopathological examination that depends on the presence of granulomatous inflammation and causes of necrosis. However, histology does not distinguish between EPTB and infections from other granulomatous diseases such as NTM, sarcoidosis, leprosy, and systemic lupus erythematosus [15].

The tuberculin skin test is also useful for the diagnosis of EPTB. However, false-positive reactions occur as a result of previous Bacille Calmette Guerin (BCG) vaccination or sensitization

to NTM, and false-negative results that occurred in the immune-compromised patients, elderly persons or overt forms of TB [12]. Moreover, in vitro T-cell-based interferon-gamma release assays have been used for the diagnosis of both latent and active TB, but these assays do not differentiate between latent and active TB infection [14].

Nucleic acid amplification tests (NAATs) require an expensive thermal cycler to amplify DNA fragments in multiple temperature-dependent steps. Therefore, some polymerase chain reaction (PCR) assays, such as Xpert MTB/RIF, are very costly, which is an obstacle to use in low-income countries. Loop-mediated isothermal amplification (LAMP) is also the molecular techniques used for TB diagnosis. It is an isothermal DNA method that relies on two or three sets of primers to amplify minute quantities of DNA within a shorter period of time. Compared with other NAATs, LAMP is cheap [15]. LAMP is a new assay with high accuracy for pulmonary TB detection [16]. However, in EPTB its accuracy varies from site to site of the organs where the disease manifested and LAMP has good diagnostic efficacy in EPTB detection [10]. In resource-limited countries, spoligotyping is remained an important tool to analyze the frequency of various *MTB* genotypes strains. It is a PCR based method for detection and typing of the MTB complex using a single chromosome locus with high polymorphism and the direct repeat (DR) region. It consists of a sequence of 36 pb which are separated by 43 non-repeated spacers. Spoligotyping offers great advantages such as identifying different strains which shows unique hybridization pattern and strains of an outbreak shared the same pattern [17]. The patterns obtained from direct examination of the clinical samples are identical to the pattern obtained from cultures of the same samples by DNA sequencing [18].

Genotype MTBDR*plus* and MTBDRsl are molecular-based assays designed to detect specific drug resistance encoding mutations in MTB have the advantage of achieving results within 48 hours, much faster than conventional drug susceptibility test (DST). The Hain line probe assays use reverse hybridization technology to detect mutations associated with resistance of tubercle bacilli to both first- and second-line anti-TB drugs. Genotype MTBDR*plus* allows for identification of Isoniazid (INH) and rifampicin (RIF) resistance by disclosing mutations in the katG, inhA, and rpoB genes, while MTBDRsl detects resistance to fluoroquinolones (FQs), aminoglycosides (kanamycin (KAN); amikacin (AMK); viomycin (VIO) and capreomycin (CAP)) by finding mutations in four different loci such as gyrA, gyrB, rrs and eis [19].

1.2. Statement of the problem

There are an estimated 10 million new cases of TB and an estimated 1.2 million people died in 2018 [2]. Multidrug-resistant TB (MDR-TB) remains a public health crisis and a health security threat across the world. World Health Organization (WHO) estimate indicated that 558 000 new cases of rifampicin resistance occurred in the globe and 82% of rifampicin-resistant cases develop MDR-TB. Moreover, about 9% of MDR-TB patients also have extensively drug-resistant TB (XDR-TB) [3]. The proportion of EPTB among all TB cases varies from country to country. Previous reports have shown that the proportions of EPTB in new TB cases are ranged from 17% to 52% across the world [20]. Moreover, the proportion of EPTB in both new and relapse TB cases in South Asia countries (Afghanistan, Pakistan, India, and Bangladesh) ranged from 19% to 23% [21].

Treating MDR-TB is more complex than treating drug-sensitive TB, as second-line TB drugs are more difficult to acquire, often require intravenous administration, and are more toxic and less effective than first-line TB drugs (2). Patients with MDR-TB also require longer progressions of more costly treatment and experience higher mortality than those infected with drug-sensitive TB (3). Moreover, it can take two years or more to treat MDR-TB, resulting in social isolation, loss of employment, and long-term socioeconomic and psychological effects

The primary drug-resistance in EPTB cases may be due to infection with primary drug-resistant strains spread out by pulmonary tuberculosis patients. Though EPTB does not spread from one patient to the other posing a threat of spread with primary drug-resistant strains, it causes serious complications unless diagnosed and treated earlier [8]. There are two ways by which people get drug-resistant TB. First, when TB treatment is inadequate, i.e., when patients fail to adhere to the treatment regimens and wrong drug prescription. Second, when there is a direct transmission of drug-resistant TB bacilli from one person to another and co-infection are increases drug-resistance [6]. Diagnosis of EPTB is difficult due to the paucibacillary nature of the disease (low bacterial load in the non-respiratory specimens), challenging sample collection, variable clinical presentation, and invasive procedures to obtain an appropriate sample and lack of laboratory facilities in resource-limited settings [9, 10].

TB is a major public health problem in Ethiopia. Ethiopia is one of the 30 high multidrug resistance TB (MDR-TB) countries and ranked 15th with more than 5,800 estimated new cases

each year [3]. A recent estimate indicated that the prevalence of MDR-TB is 0.71% in newly diagnosed patients and 16% in previously treated patients in Ethiopia [2]. In addition, a recent systematic review of local MDR-TB prevalence studies in Ethiopia included six eligible papers for analysis out of 23 studies done between1994-2012 and reported a high overall MDR-TB prevalence in the range of 3.3%-46.3%. Also, two studies done in Bahir Dar (northwest Ethiopia) in 2011 and Addis Ababa in 2005-2006 reported 1% and 4.4% XDR-TB respectively [6]. Besides, the prevalence of culture-confirmed extrapulmonary tuberculosis is 29.8% among extrapulmonary tuberculosis suspected cases in Ethiopia. The proportion of EPTB, with the majority of TB lymph nodes among newly diagnosed TB patients, has been increasing for the last two decades [22]. Despite the high burden of EPTB in Ethiopia, there is an information gap on drug resistance patterns and molecular characterization of EPTB. Thus, the current study was conducted to determine molecular characterization and drug resistance patterns of MTB complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia.

1.3. The rationale of the study

The information about molecular characteristics and DST of MTB among EPTB in Addis Ababa is limited. There is no strong evidence on the burden of drug resistance and molecular characteristics among EPTB patients in Ethiopia. The information is very crucial to provide an overview of molecular characteristics of MTB causing EPTB which is useful to understand the new strains that circulate in the population. So far only one study conducted in Addis Ababa using spoligotyping and Geno-Type MTBC to identify the strain and drug resistance [23]. However, in this study, LPA was performed to identify mutation type associated with drug-resistance for all culture-positive EPTB isolates, and further second-line anti-TB drug resistance was determined for RR/MDR EPTB isolates using SL-LPA, and phenotypic DST. Also, the above study has included participants from two hospitals only, but the current study was covered participants from eight hospitals which makes the current study more generalizable than the previous study (the sample size of the EPTB isolates three-fold of the previous one). The molecular characteristics among strains of MTB have a great effect on the pathogenicity and transmissibility, design for vaccines production, identification of genes for drug targets, and improving molecular diagnostic techniques. Therefore, this study was aimed to determine drug resistance patterns and molecular characteristics of MTB among confirmed extrapulmonary tuberculosis cases in Addis Ababa.

2. Literature review

A study reported from Brazil by Gomes *et al* on 612 isolates of EPTB cases indicated 506 (83%) SIT level, while 106 (17%) had orphan patterns, 297 (48%) belonged to the Latin-American Mediterranean family (LAM) family, 82 (13%) belonged to the T family and 45 (7%) belonged to the Haarlem family [24]. A cross-sectional study reported from India by Suzana *et al* is also indicated that 61 spoligo patterns, 128 clusters in the spoligotype database (spoldb4 database) with spoligo international type (SIT) number and 35 true unique isolates [25]. The most predominant spoligotype identified was EAI lineage (56) which followed by Beijing (28), CAS (20), T (9), U (7), X (3), H (3), BOVIS 1 BCG (1) and LAM (1) [25]. Out of the 163 isolates tested for resistance only 42 of the 163 MTB isolates had MDR and 25 Pre-XDR [25].

Similarly, another study reported from India by Kandhakumari *et al* revealed that 67 *Mycobacterium tuberculosis* isolated from a single EPTB specimen. Among 67 isolates with 41 spoligo patterns, 28 isolates (41.8%) were EAI lineage which was the predominated and followed by 18 orphans (26.9%), 10 Beijing (14.9%) and 8 U (11.9%) [26]. However, BOVIS1 BCG (ST482), T1-T2 (ST78), and H3 (ST50) strain each were represented by a single strain [26]. Still another study reported by Sankar *et al* from North India shown that 125 MTB strains isolate from patients with EPTB [17]. The spoligotyping results indicated that 110 (88%) displayed known patterns while 15 (12%) isolates had no matching database [17]. CAS family (57.27%) was the most predominant spoligotype that isolated. Moreover, 38 isolates were the largest clade and belonging to the CAS1 DEL lineage [17]. Thus CAS family lineage is the most prevalent genotype in the EPTB cases in the population [17].

The study reported from Italy indicated the association of MTBC isolates of BOVIS and CAS genotypic lineages with EPTB from 244 isolates [27]. Spoligotyping identified were ten major genotypic families, such as Africanum (1.4%), Beijing (7.2%), Bovis (1.7%), CAS (2.7%), EAI (4.5%), Haarlem (21.4%), LAM (12.8%), S (5.2%), T (28.2%), X (1.2%), and other spoligotypes undefined in SpolDB4 (13.8%) [27]. The distribution of EPTB among the genotype families was reported [27]. The study reported from Turkey also indicated that spoligotyping identified a total of nine spoligo superfamilies that include 344 (86.6%) of the 397 study isolates [28]. Fifty-three (13.4%) of the spoligotype was not matched to any spoligotypes in the SpolDB4 database [28]. Ill-defined T (46.3%, 184/397), LAM, (19.4%, 77/397), and Haarlem (H, 14.1%, 56/397) were

the most commonly seen super families reported in the same study [28]. Still another study reported from Turkey indicated that 132 different spoligopatterns were identified and 46 different clusters for 384 strains were determined [29]. The most predominant spoligotypes were ST53 (24.7%) and ST41 (8.1%) which followed by ST50 (5.7%), ST284 (4.7%), and ST4 (4.3%), respectively [29]. ST53 was the most predominant type in both sexes [29]. MDR was determined in 12 isolates, of which six were ST1 [29]. DST was applied for 396 patients and 14 strains showed resistance to RIF, with high-level resistance to INH in 8, low-level resistance to INH in 20, and MDR in 12, of which six were type ST1 [29]. The overall MDR proportion was 3% [29].

A study conducted in Mozambique has indicated 45 isolates were *Mycobacterium tuberculosis*, but none of *Mycobacterium bovis* was identified [30]. Cervical TBLN comprises 39 (86.7 %) of cases, which is the main cause of TBLN, and 66.7% of those cases were identified from people living with HIV [30]. Moreover, the result reported from Maputo a capital of Mozambique indicated that majorities of TBLN caused by a variety of *Mycobacterium tuberculosis* strains [30]. The most prevalent lineage identified was EAI 19 (43.2 %) [30]. The specific common spoligotypes identified were SIT 48, SIT 42 (LAM 9), SIT 1 (Beijing), and SIT53 (T1) [30].

The study reported by Wamala *et al* indicated clinicopathological features of TB due to *M. tuberculosis* Uganda genotype in patients with TB lymphadenitis [31]. Spoligotyping revealed that 54 distinct spoligotype patterns of which 33 (61% patterns were available in the SITVIT-WEB and 21(39%) patterns were not found in the database [31]. Moreover, Euro-American lineages were predominant (62%) which followed by CAS lineage (18%) [31]. There were also three Bejing strain and not *Mycobacterium bovis* [31].

A study reported from Ethiopia on molecular and drug sensitivity patterns of mycobacterial isolates from EPTB cases indicated that, out of 200 clinically suspected extrapulmonary tuberculosis patients, only one was *M. bovis* and 58 were MTB strains with 31 different spoligotype patterns grouped into seven clusters [23]. Euro American lineage (71.1 %), Indo Oceanic (5.1 %), East Asian (3.4 %) East Africa Indian (18.6 %) and *M. bovis* (1.6 %) [23]. Resistance to RIF was higher (22 %) than resistant to INH, streptomycin (STM) and Ethambutol (EMB) (8.1 %, 5 %, and 3 % respectively) [23]. Out of the 37 isolates tested for resistance, only 2 isolates were resistant for both STM and INH, but no MDR strain was found [23]. Moreover, a study reported from Dessie Hospitals, Northern Ethiopia identified 28 different strains [32]. Two

of these strains were SIT50 and SIT393 that consisted of three isolates each, but the remaining 26 strains were an orphan strain [32]. Classifying strains on the bases of phylogeny level (lineage) of MTB using SPOTCLUST software revealed that the strains belonged to Euro-American (EA) 57.1% (16/28), East-African Indian (EAI) 14.3% (4/28) and Indio Oceanic (IO) 28.6% (8/28) lineages [32]. A similar study reported from Debre Birhan Hospital from central Ethiopia revealed 16 clusters out of which 2 were new to the SITVIT database [33]. Of the 44 spoligotypes, 27 (61.4%) were from modern (Euro-American) and 16 (36.3%) were ancestral (Indo-Oceanic) tuberculosis lineages [33]. The most dominant spoligotypes were SIT54, SIT53, and SIT149 in decreasing order [33]. SIT54, SIT134, SIT173, SIT345, SIT357, SIT926, SIT91088, and SIT1580 were reported for the first time in Ethiopia [33]. The family with the highest frequency identified as MTB family was T1 [33]. Most of the strains belonged to EA and IO lineages [33].

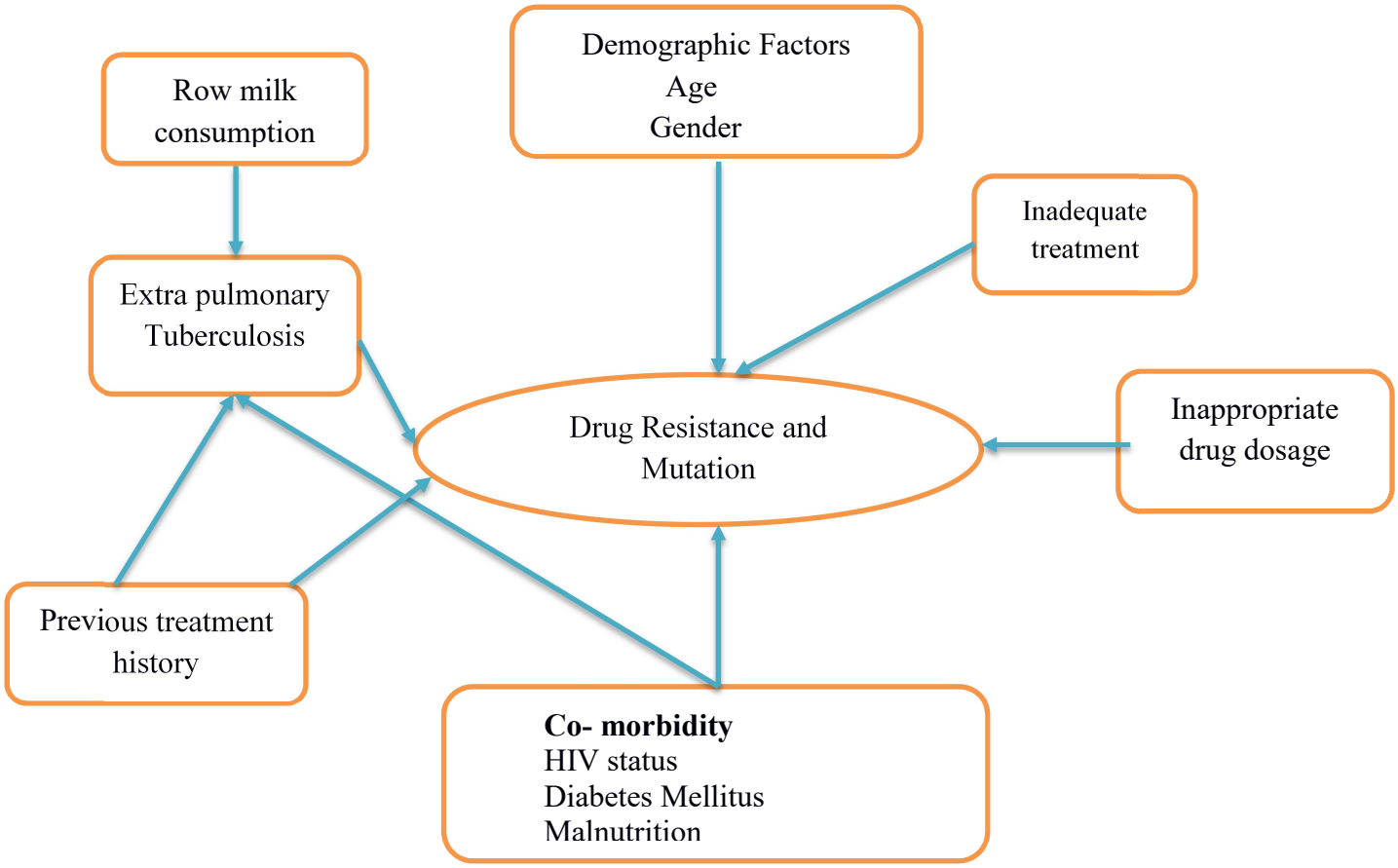


Figure 1: Conceptual framework of a risk factor for extrapulmonary tuberculosis and drug resistance [3, 8, 9.23, 24, 29, 32, 31, 33].

3. Objectives

3.1. General objective:

The main aim of this study was to determine molecular characterization and drug resistance patterns of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis isolates in Addis Ababa, Ethiopia.

3.2. Specific objectives

- To determine the drug susceptibility profile of *Mycobacterium tuberculosis* complex in isolates obtained from extrapulmonary tuberculosis patients.
- To describe the molecular characterization of *Mycobacterium* species
- To determine the frequency of gene mutations associated with drug resistance of *Mycobacterium tuberculosis*.
- To compare the performance of LPA with MGIT 960 for the detection of drug resistance in extrapulmonary tuberculosis.

4. Hypothesis

Regarding variation in the molecular characterization profiles, there was no difference, but regarding with drug resistance patterns of *Mycobacterium tuberculosis* isolates of EPTB between the current study and a research done in Addis Ababa and Debrebirhan showing difference the burden of drug resistance in EPTB

5. Materials and Methods

5.1. Study area and study site

This study was conducted to analyze the molecular characteristics of MTBC among confirmed EPTB cases in Addis Ababa, Ethiopia. Addis Ababa is the capital city of Ethiopia which covers an area of 527 square kilometers. The city has estimated the total population of 3,384,569 according to the 2007 census [34]. Eight public hospitals such as Saint Paul Millennium Medical College hospital, ALERT Hospital, Armed Force Hospital, Black Lion Hospital, Ras Desta Damto Memorial Hospital, Zewiditu Memorial Hospital, Saint Peter Hospital, and Yekatit 12 Hospital were included in this study. Laboratory analysis was done at the National Tuberculosis Reference Laboratory (NTRL), Ethiopian Public Health Institute (EPHI). This laboratory operates under a biosafety level III based on the WHO recommendation for TB culture and DST tests. Among the tests at the NTRL TB culture, Phenotyping DST, Genotyping DST (Line Probe Assay and Xpert MTB/RIF Assay) tests, spoligotyping, and Geno-Type MTBC techniques are available for this research work.

5.2. Study design and Period

A laboratory-based cross-sectional study was conducted among confirmed EPTB cases in Addis Ababa, Ethiopia in a period between November 2019 and April 2020. Laboratory analysis was done at NTRL, EPHI. This study was the continuation of two studies; a study from the evaluation of Xpert MTB/RIF Assay for the diagnosis of EPTB [Unpublished data], and a study on the diagnostic accuracy of Xpert MTB/RIF assay and non-molecular methods for the diagnosis of tuberculosis lymphadenitis [35] conducted from January 2016 to August 2017.

5.3. The study population

Isolates were collected from patients diagnosed as EPTB cases in between January 2016 to August 2017 at eight referral hospitals in Addis Ababa, Ethiopia. The isolates were properly stored in a deep freezer (-80◦C) at NTRL of EPHI for further study.

5.4. Inclusion and Exclusion criteria

5.4.1. Inclusion criteria

- All culture-positive isolates presented with a correct patient identification number and had demographic data were included in this study.

5.4.2. Exclusion criteria

- NTM and contaminated EPTB isolates were excluded from this study.

5.5. Study Variables

5.5.1. Dependent variables

- MTB strains type, mutation type, and drug resistance profile

5.5.2. Independent variables

- Demographic variables such as age, sex, marital status, educational status, previous TB treatment history, diabetes mellitus status, and HIV serostatus were independent variables

5.6. Measurement and Data collection

5.6.1. Sample Size Determination

In this study, we have included all the available 151 isolates stored at EPHI laboratories

5.6.2. Sampling method

Special sampling technique was not used rather; all 151 available stored isolates that fulfilled the inclusion criteria were included in the study.

5.6.3. Sub-culturing of stored isolates collection

All culture-positive isolates were obtained from EPTB patients attended eight referral hospitals in Addis Ababa during the previous study pried were included in this study. These samples from which the culture-positive isolates obtained were collected as part of the routine diagnostic examination of suspected EPTB patients treated in the referral hospitals indicated above. Informed consent for the use of *MTB* isolates was obtained from each patient. Also, as part of the

routine clinical examination, information on the current and previous history of *MTB* infection, history of contact with TB case and history of receiving previous anti-tuberculosis treatment was extracted from laboratory request forms. Extra-pulmonary specimens were collected from 778 clinical presumptive EPTB patients for the evaluation of Xpert MTB/RIF Assay for the diagnosis of EPTB that was conducted in 2017 study and 152 clinical presumptive EPTB cases for Diagnostic accuracy of Xpert MTB/RIF assay and non-molecular methods for the diagnosis of tuberculosis lymphadenitis in 2016 study. All specimens were tested for MTBC by culture and Xpert MTB/RIF for both studies. Of 930 extrapulmonary samples, 151 (16.2%) culture-positive isolates were obtained. Thus, 151 stored isolates were used in this study. All stored isolates were sub-cultured in liquid culture media for the detection of *mycobacterium tuberculosis*.

5.6.4. Laboratory analysis

5.6.4.1. First-line phenotypic drug susceptibility test

Five first-line drugs such as streptomycin (STM), isoniazid (INH), rifampicin (RIF) ethambutol (EMB), and Pyrazinamide (PZA) were tested using Mycobacterium Growth Indicator Tube 960 (MGIT 960) system. DST was performed by Antibiotic Susceptibility Testing (AST) set with the proportional method recommended by the WHO (36). The concentrations of the drugs in media were: STM 1.0μg/mL, INH 0.1μg/mL, RFP 1.0μg/mL, EMB 5μg/mL and PZA 100μg/mL which consists a growth control tube for each drug contain tube. The bacterial inoculums were diluted to 1:100 before inoculation into the growth control tube and 0.5 mL bacterial suspension was added (37). The inoculated tubes were incubated in MGIT 960 system and monitored every one hour for an increase in fluorescence. For SIRE sensitivity MGIT 960 tubes were incubated for a maximum of 13 days and 21 days for PZA. [36].

5.6.4.2. Second-line phenotypic drug susceptibility test

Second-line DST was performed for all MDR-TB isolates using MGIT 960 systems. All liquid MGIT-positive MTB culture within 1 to 5 days were used for second-line DST. 800µl SIRE supplement and 100µl working drug solution were added into MGIT tube which contained 7ml modified Middlebrook 7H9. A working solution of each drug was prepared at the concentration level of: Ofloxacin (OFX) 2.0µg/ml, Capreomycin (CAP) 1.25µg/ml, Amikacin (AMK)

1.0µg/ml, Kanamycin (KAN) 2.5µg/ml, and Ethionamide (ETH) 2.5 µg/ml based on the manufacturer's recommendations [37, 38].

5.6.4.3. Geno-Type MTBDR*plus* and MTBDR*sl* assay

The MTBDR*plus* and the MTBDR*sl* assays were performed directly on the MTB isolates according to the manufacturer's instructions. The first DNA was extracted from *Mycobacterium tuberculosis* complex isolates. Next, PCR was performed using pre-made amplification mixes (amplification mix A and amplification mix B) that contained all the necessary components. Following amplification, DNA was hybridized with specific oligonucleotide probes immobilized on a strip which enables the detection of the presence of MTBC as well as simultaneously the presence of wild-type and mutation probes for resistance to RIF, INH, FQs and injectable drugs (CAP, AMK, VIO, and KAN). If a mutation is present in one of the target regions, the amplicon was not hybridized with the relevant probe. Mutations can be detected by a lack of binding to wild-type probes as well as by binding to specific mutation probes for the most commonly occurring mutations. Visualization was achieved using a streptavidin-alkaline phosphates conjugate-mediated colorimetric reaction leading to colored bands on the strip at the site of probe binding which can be read with the naked eye [19, 39, 40, 41].

5.6.4.4. GenoType MTBC

Differentiation of MTBC down to the species level was carried out by using genotype MTBC assay. Geno-Type MTBC is a molecular Genetic Assay for the differentiation of the MTBC from cultured materials. It is a qualitative in vitro test from cultured materials for the identification of species or strains belonging to the MTBC. The complexes are *M.tuberculosis/M.canettii, M.bovis, M.africanum, M.microti, Sub-species of bovis, M.caprae, M.bovis BCG.* The procedure involved in Genotype MTBC Hain molecular line probe assay includes the following method DNA Extraction, DNA Amplification, and Hybridization of the amplified DNA and these were done according to the manufacturer instructions [42].

5.6.4.5. Spoligotyping of MTBC

Spoligotyping of Mycobacterium tuberculosis Complex Isolates was carried out by use of Ligation-Based Amplification and Melting Curve Analysis. Spoligotyping was performed as described elsewhere [43]. Briefly, 5µl suspension of heat-killed mycobacterial cells was added

into 20µl a reaction mixture (a total volume of 25 µl) which contains 12.5 µl HotStar Taq Master Mix (Qiagen), 2 µl each primers (DRa and DRb), 3.5 µl molecular grade water and 5 µl suspension of heat-killed cells. The mixture was amplified DNA by PCR and heated for 10 minutes at 96^{o}C. The PCR products are labeled with biotin because of DRa primer was biotinylated. Then 25 µl of the amplified PCR product was mixed with 150µl primary buffer, loaded on the minibloter and hybridized for 60 minutes at 60^{o}C in the standard hybridizing oven to a set of 43 immobilized oligonucleotides, each cross ponding to one of the unique spacer DNA sequences within the directed repeat locus. After hybridization, the membrane was washed twice in 250 ml seconder buffer at 60^{o}C for 10 minutes on the shaking platform. Then, following by cooling the membrane in distilled water at roller bottle and add 7µl streptavidin peroxidase-conjugated for 60 minutes incubate at 42^{o}C in hybridizing oven. After this stapes, the membrane was washed twice in 250 ml by seconder buffer for 10 minutes at 42^{o}C by shaking which was followed by two times washing the membrane using 250 mL 2X SSPE (Saline-Sodium phosphate EDTA buffer) for 5 minutes at room temperature. Finally, Hybridized DNA was detected by the enhanced chemiluminescence detection system and by exposure to X-ray film per specification of the manufacturer and absence or presence of spacers was detected by visualizing the black spots developed on the membrane film [44, 45].

5.7. Data Quality Assurance

Quality was assured by using different approaches. Trained laboratory professionals were performed the molecular and drug susceptibility tests. Internal quality control was analyzed along with study clinical isolates. A known susceptible *MTB (H37Rv)* control and resistant isolate were tested by including them in each test run of drug susceptibility test for molecular characterization known susceptible *MTBC* (*H37Rv* and *BCG)* were run at each batch of the test as control. The sterility of the culture media was checked and a 10% confirmed result was repeated for all methods. Double data entries were done and data cleaning was made before the main analysis.

5.8. Data Analysis and Interpretation

The spoligotype patterns obtained were entered into the international spoligotyping database, and assign into the existing SIT (Spoligotype International Type) number. All data generated from the laboratory result were double entered using into SPSS version 23 statistical software. A

descriptive phenotypic, genotypic and epidemiologic data analysis was done. The level of statistical significance was set at $p \leq 0.05$.

5.9. Ethical consideration

Ethical clearance was obtained from the department of Research and Ethical Review Committee of Medical Laboratory Sciences, Addis Ababa University. The letter of collaboration with ethical clearance was submitted to EPHI to getting permission to use the required information and stored isolate. The study was conducted by stored clinical isolates anonymously, and confidentiality of the results was assured by keeping the documents in a locked area. The name of the client did not appear on the data collection template.

5.10. Dissemination of Results

After conducting the research, the results of the study were submitted to Addis Ababa University, College of Health Sciences and the Department of Laboratory Sciences. Also, the result of this study was presented as a technical report or orally to EPHI and other concerned bodies. The finding of the study was also presented to the medical scientific community and a manuscript was submitted to peer-reviewed journals for publication.

5.11. Operational Definitions:

Multidrug resistance (MDR): Resistant to at least isoniazid and rifampin, the two most potent TB drugs [2].

Pre-extensively drug-resistant TB (pre-XDR-TB): resistance to isoniazid and rifampicin and either a FQ or a second-line injectable agent but not both.

Extensively drug-resistant TB (XDR TB): resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs [19].

Extrapulmonary tuberculosis (EPTB): a case of TB that involves organs other than the lung e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones, and meninges [17].

6. Results

6.1. Demographic data of study participants

Of 151 isolates from EPTB patients included in this study, more than half of the study participants (54.3 %) were males. The mean age of the participants was 32.3 years (SD 17.32 years) with an age range of 4 to 78 years. The majority of participants were in the young-age adult groups 15 to 39 years. Most of the patients (90.7 %) had no previous TB treatment history, and 51.0% were attended primary school, 44.9% married and 30.5% were daily laborer. About 22.5% of the participants were HIV sero-reactive, and 9.3% had diabetes mellitus [Table 1].

Table 1: Demographic data of EPTB patients in (n=151), 2020

Characteristics		Frequency	Percentage (%)
Age in years	<15	16	10.6
	15-39	89	58.9
	40-59	33	21.9
	>60	13	8.6
Gender	Female	69	45.7
	Male	82	54.3
HIV status	Nonreactive	96	63.6
	Reactive	34	22.5
	Unknown	21	13.9
Marital status	Married	98	64.9
	Single	44	29.1
	Separated	9	6.0
Educational status	Illiterate	42	27.8
	Primary School	77	51.0
	Secondary School	24	15.9
	Higher Education	8	5.3
Occupation	House wife	13	8.6
	Daily laborer	46	30.5
	Government employee	12	7.9
	Unemployed	38	25.2
	Other	42	27.8
Diabetes Mellitus	Yes	14	9.3
	No	137	90.7
MDR-TB contact	Yes	7	4.6
	No	144	95.4
TB Category	New cases	137	90.7
	Re-treatment	14	9.3

*TB-Tuberculosis, *MDRTB-Multidrug Resistance Tuberculosis and *HIV-Human Immunodeficiency Virus

Figure 2 depicts the organ distribution of EPTB in 151 isolates included in this study. Based on the clinical presentation frequency distribution of different types of specimen for EPTB, 77.8 % of the enrolled patients fall into two major disease categories; 99 (65.6 %) tuberculous lymphadenitis or Lymph node aspirate, 32 (21.2 %) pleural tuberculosis

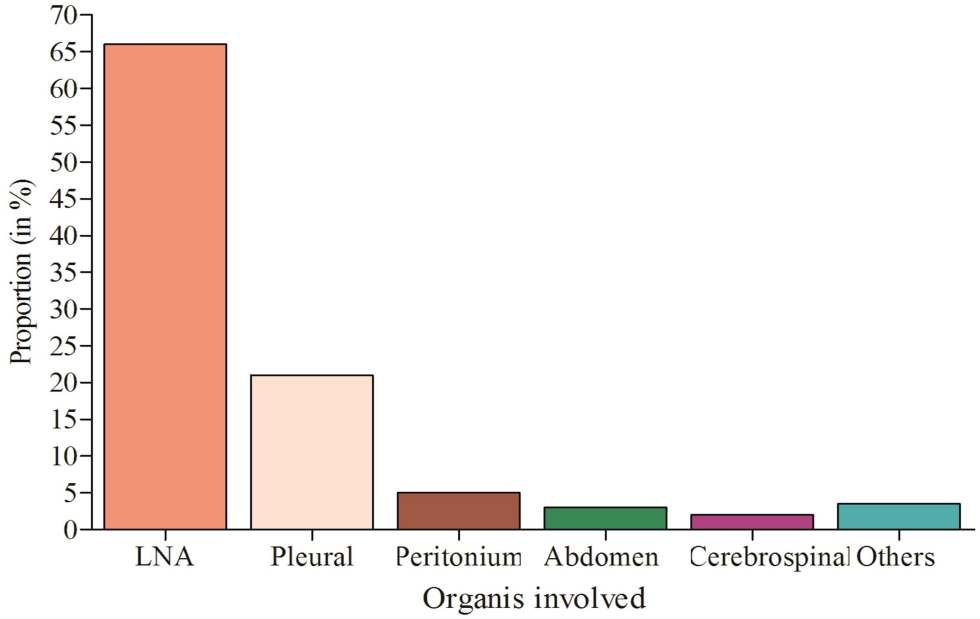


Figure 2: Frequency of different types of specimen for EPTB.

6.2. Phenotypic Drug sensitivity pattern of Mycobacteria for the first line

A total of 151 isolates from EPTB specimens were performed phenotypic drug susceptibility testing by BACTEC MGIT 960 for first-line drugs. Of 151 isolates, 85.4% (129/151) were sensitive to all first-line drugs: STM, INH, RIF EMB, and PZA. However, twenty-nine isolates were resistant to at least one or more drugs. Twenty-two (14.6%) of isolates were resistant to INH and similar numbers were resistant to PZA. Similarly, 14 (9.3%) of isolates were resistant to RIF, while nine (6.0%) resistant to STM and three (2.0%) were resistant to EMB. The overall proportion of MDR-TB was 9.3%. A higher proportion of MDR-TB was seen in previously treated TB cases than new cases [Table 2].

Table 2 Phenotyping drug resistance profiles for first-line drugs among confirmed EPTB isolates by Patient History profile, 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n %	Total n (%)
Total tested Isolate	137	14	151
Susceptible	118(86.1)	11(78.6)	129(85.4)
Resistance to any drug			
STM	7(5.1)	2(14.2)	9 (6.0)
INH	19(13.8)	3(21.4)	22 (14.6)
RIF	11(8.0)	3(21.4)	14 (9.3)
EMB	3(2.2)	-	3 (2.0)
PZA	19(13.9)	3(21.4)	22(14.6)
Mono resistance			
INH	2(1.5)	1(7.1)	3 (2.0)
PZA	8(5.8)	1(7.1)	9(5.9)
Resistance to more than one drug			
INH + RIF	11(8.0)	3(21.4)	14(9.3)
INH + PZA	12(8.6)	3(21.4)	15(9.9)
INH + STM	7(5.1)	2(14.3)	9(6.0)
INH + EMB	3(2.2)	-	3(1.32)
INH + RIF + STM	4(2.9)	1(7.1)	5(3.3)
INH + RIF + EMB	2(1.5)	-	2(1.3)
INH + RIF + PZA	8(5.8)	2(14.3)	10(6.6)
INH + RIF + EMB + PZA	1(0.7)	-	1(0.7)
STM + INH + RIF + PZA	4(2.9)	1(7.1)	5(3.3)
INH + RIF or MDR	11(8.0)	3(21.14)	14 (9.3)

*STM-Streptomycin *INH-Isoniazid * RIF-Rifampicin * EMB-Ethambutol * PZA-Pyrazinamide and *MDR-Multiple drug resistance

6.3. Second-line phenotypic drug sensitivity testing

Second-line drugs DST were conducted for a total of 14 MDR isolates. The second-line drugs for which DST conducted in this study include AMK, CAP, ETH, KAN, MOX, and OFX. Of 14 isolates 14.3% were resistant to MOX and 7.1% to CAP. Similarly, of 14 isolates 7.1% were resistant to ETH, while 14.3% resistant to OFX. Of 14 MDR-TB isolates, 21.4% were developed pre-XDR-TB. Of the three isolates that developed pre-XDR-TB, a high proportion (33.3%) was observed in previously treated cases while 18.2% (2/11) in new cases. Out of the 14 isolates, none of the cases had XDR-TB in the present study [Table 3].

Table 3: Second-line phenotypic drug sensitivity testing according to new cases and previously treated cases (n = 14), 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n (%)	Total n (%)
Total Isolate	**11**	**3**	**14**
Amikacin	-	-	-
Capromycin	1(9.1)	-	1 (7.1)
Ethionamide	1(9.1)	-	1(7.1)
Kanamycin	-	-	-
Ofloxacin	1(9.1)	1(33.3)	2 (14.3)
Moxifloxacin	1(9.1)	1(33.3)	2 (14.3)
Pre-XDR	2(18.2)	1(33.3)	3 (21.4)

*XDR-Extensively Drug Resistant

6.4. First Line Genotypic Drug Resistance Prediction

Of 151 isolates on which genotype MTBDR*plus* assay performed 130 (86.1%) were sensitive to INH and RIF. However, 20 (13.2%) isolates were resistant to INH, while 15 (9.9%) resistant to RIF. Out of 151 isolates, 14 (9.3%) were developed MDR-TB and 21 (14%) developed resistance to any drug by GenoType MTBDR*plus* assay [Table 4].

Table 4: The proportion of genotyping drug resistance from a total of 151 *Mycobacterium tuberculosis* complex isolates from EPTB patients, 2020

Characteristics	Frequency	Percentage
Susceptible	130	86.1%
INH resistance	20	13.2%
RIF resistance	15	9.9%
Any drug resistance TB	21	14.0%
MDR-TB	14	9.3%

*RIF-Rifampicin, *INH-Isoniazid, *MDR-Multiple drug resistance and *TB-Tuberculosis

Of the total of 151 isolates, 21 were found either RIF or INH resistant. An rpoB mutation indicates that 15 (9.9%) were resistant to RIF. The RIF resistant isolates were shown mutations at different amino acid positions. Of 151 isolates, 13 (8.6%) had an amino acid mutation at the S531L position, and one (0.7%) isolates had at H526Y position. Of a total of RIF resistant isolates, two isolates had mutations in codon 526-529 that indicated the absence of wild type band (WT7) and 13 isolates had mutations in codon 530–533 which show the absence of wild type band (WT8). Mutations in katG and inhA genes lead to INH resistance. Of a total of 151 isolates, 18 (11.9%) had amino acid mutation at the S315T1 position and one (0.7%) had at C15T position. Among the INH resistant isolates, a missed wild-type probe was observed only on one isolate at inhA WT1 [Table 5].

Table 5: Frequency of gene mutations associated with resistance to Rifampicin (rpoB) and Isoniazid (katG and inhA) in EPTB isolates by Genotype MTBDR*plus* assay (n=151), 2020

Gene	Band	Mutant probe	Number of strains (n)	Percentage (%)
rpoB				
	WT1	506-509	-	-
	WT2	510-513	-	-
	WT3	513-517	-	-
	WT4	516-519	-	-
	WT5	518-522	-	-
	WT6	521-525	-	-
	WT7	526-529	2	1.3%
	WT8	530-533	13	8.6%
	MUT1	D516V	-	-
	MUT2A	H526Y	1	0.7%
	MUT2B	H52D	-	-
	MUT3	S531L	13	8.6%
katG				
	WT	315	18	11.9%
	MUT1	S315T1	18	11.9%
	MUT2A	S315T2	-	-
inhA				
	WT1	-15	1	0.7%
	WT2	-8	-	-
	MUT1	C15T	1	0.7%
	MUT2	A16G	-	-
	MUT3A	T8C	-	-
	MUT3B	T8A	-	-

* MUT-mutant and *WT-wild type.

6.5. Second Line Genotypic Drug Resistance Prediction

Fifteen (9.9%) RR-TB strains were identified among the 151 *M. tuberculosis* isolates used for first-line DST. To determine the number and percentage of pre-XDR and XDR strains, we performed DST for second-line drugs on all available MDR strains in this study. Of the total 15 MDR-TB or RR-TB isolates, two isolates were resistant to fluoroquinolone drugs and only one isolate was resistant to the injectable drugs alone. Of 15 isolates three (20%) isolate was identified as pre-XDR strains and XDR strain was not found from MDR-TB [Table 6].

Table 6: The proportion of second-line drug resistance by genotyping from EPTB patients (n=15), 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n (%)	Total n (%)
Total Isolate	**12**	**3**	**15**
Injectable drug	1(8.3)	-	1(6.6)
Fluoroquinolone drug	1(8.3)	1(33.3)	2 (13.3)
Pre-XDR	2(16.6)	1(33.3)	3 (20)
XDR	-	-	-

*XDR-Extensively Drug Resistant

The analysis of gyrA gene on 15 RR-TB isolates was revealed that one (6.6%) isolates had a mutation at codon A90V, while another one (6.6%) had a mutation at codon D94G, (6.6%) had a mutation at codon D94H, while another one (6.6%) had variable mutations at codon S91P. In gyrA gene analysis the most frequent mutations were the absence of bands WT3 in three (20%) and WT2 in two (13.3%) isolates. Other mutations were assessed in rrs gene for second-line injectable drug resistance. The rrs gene analysis was revealed that one (6.6%) isolates had a mutation at codon a1401g and the absence of bands WT1 in one (6.6%) isolates [Table 7].

Table 7: Frequency of gene mutations associated with resistance fluoroquinolone resistance (gyrA) and injectable drug resistance (rrs) by Genotype MTBDRsl assay (n=15), 2020

Gene	Band	Gene region or mutation	Number of strains (n= 15)	Percentage (%)
gyrA				
	WT1	85–89	-	-
	WT2	89–93	2	13.3%
	WT3	92–96	3	20%
	MUT1	A90V	1	6.6%
	MUT2	S91P	1	6.6%
	MUT3A	D94A	-	-
	MUT3B	D94N or D94Y	-	-
	MUT3C	D94G	1	6.6%
	MUT3D	D94H	1	6.6%
gyrB				
	WT1		-	-
	MUT1	N538D	-	-
	MUT2	E540V	-	-
Rrs			-	-
	WT1	1400	1	6.6%
	WT2	1484	-	-
	MUT1	A1401G	1	6.6%
	MUT2	G1484T	-	-
Eis				
	WT1	-37	-	-
	WT2	-10 to -14	-	-
	WT3		-	-
	MUT1	C-14T	-	-

Note: Data are presented as *MUT = mutant; *WT = wild type.

6.6. Comparison of genotypic DST result with phenotypic DST result of *M.Tuberculosis*

Based on the analysis of drug susceptibility results by genotypic and phenotypic DST methods, of 151 total isolates three (2.0%) *MTB* isolates had discordant results between the genotypic MTBDR*plus* test and the phenotypic DST. The common pattern of disagreement was susceptible in the genotypic DST analysis, while resistant in the phenotypic DST for INH (n=2). Also, one isolate was resistant in the genotyping DST analysis, whereas susceptible to phenotyping DST for RIF (n=1). The concordances of the GenoType MTBDR*plus* assay and the MGIT DST for the detection of INH and RIF resistance were 90.9% (20/22) and 100% (14/14), respectively. Kappa agreement between INH genotypic DST and phenotypic DST was 0.95. Besides, the kappa agreement between RIF genotypic DST and phenotypic DST was 0.96. Genotypic DST was compared with phenotypic DST as a gold standard, Line probe assays showed high the sensitivity, specificity, PPV, and NPV of LPA for INH were 90.9%, 100%, 100%, 98.5%) respectively. The respective values for RIF were 100%, 99.3%, 93.3%, and 100% (Table 8).

Table 8: Comparison of genotypic with phenotypic DST of *M. tuberculosis*, 2020

GenoType MTBDRplus assay		Phenotypic MGIT DST result					
		Susceptible	Resistance	Sensitivity	Specificity	PPV	NPV
INH	Susceptible	129 (85.4%)	2 (1.3%)	90.9%	100%	100%	98.5%
	Resistance	-	20 (13.2%)				
RIF	Susceptible	136 (90.1%)	-	100%	99.3%	93.3%	100%
	Resistance	1 (0.4%)	14 (9.3%)				

*RIF-Rifampicin, *INH-Isoniazid *DST-Drug Susceptibility Test, *PPV-Positive predictive value, *NPV-Negative predictive value, *MGIT-Mycobacterium Growth Indicator Tube, *MTBDR- Mycobacterium Tuberculosis Drug Resistance

6.7. Differentiation of MTBC (GenoType MTBC) species

Out of 151 MTBC isolates, 148 (98.0%) were identified as *M. tuberculosis* while three (2.0%) *M.bovis*. Other members of MTBC such as *M. africanum* and *M. microti* were not identified [Table 9].

Table 9: Frequency of MTBC species differentiated by GenoType MTBC Assay (n= 151), 2020

MTBC species	Number	Percentage (%)
M. tuberculosis	148	98
M. africanum	-	-
M. bovis	3	2
M. microti	-	-

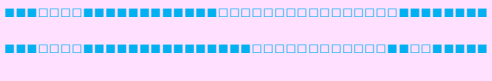
*MTBC-*Mycobacterium tuberculosis complex, *M. tuberculosis-Mycobacterium tuberculosis, *M. africanum -Mycobacterium africanum, *M. bovis-Mycobacterium bovis, *M. microti-Mycobacterium microti*

6.8. Strain typing result (spoligotyping)

All 151 MTB isolates were characterized by spoligotyping. Among these 151 MTB isolates characterized by spoligotyping, 146 (96.7%) displayed known patterns while 5 (3.3%) isolates had no matching with the international spoligotype database (SITVIT2). Of these isolates, 41 different spoligotype patterns were identified. The 146 (96.6%) isolates grouped into 11 families. The most predominant strain types were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%). The most predominant lineages were T family (55.0%), Central Asia Strain (19.2%), Haarlem (8.0%), and Orphan (2.7%). The remaining five (3.3%) isolates were not found in the database SITVIT2 and did not show similarity to any other known lineages and were considered as possibly new genotypes [Table 11].

Table 10: Spoligotyping pattern, octal codes, SIT and lineage of extra pulmonary *Mycobacterium tuberculosis* complex isolates (n = 151), 2020

Lineage /Family	SIT	No. (%)	Octal Number	Spoligotype pattern
Beijing	1	1(0.7)	000000000003771	
X1	336	1(0.7)	777776777760731	
Turkey	41	1(0.7)	777777404760771	
Haarlem		**12(8.0)**		
H1	47	1(0.7)	777777774020771	
H3	116	1(0.7)	777767775720771	
H3	121	1(0.7)	777777775720771	
H3	3	2(1.3)	000000007720771	
H3	3134	1(0.7)	777737377720771	
H3	50	2(1.3)	777777777720771	
H3	764	4(2.6)	777757777720771	
Bovis		3(2.0)		
Bovis1	694	2(1.3)	777776777760731	
Bovis1	820	1(0.7)	676763777777600	
T		**83(55.0)**		
T1	1129	2(1.3)	776777777760771	
T1	131	9(6.0)	777717777760771	
T1	393	3(2.0)	777757777760771	
T1	3315	1(0.7)	376777737760771	
T1	53	22(14.6)	777777777760771	
T2	52	2(2.3)	777777777760731	
T2	875	2(1.3)	777717777760731	
T2	Orphan	2(1.3)	017777777760731	
T3	1745	1(0.7)	773737777760771	
T3	37	6(4.0)	777737777760771	
T3-ETH	149	33(21.2)	777000377760771	
Ethiopia		**5(3.0)**		
Ethiopia	1729	1(0.7)	700000004177771	
Ethiopia	910	4(2.6)	700000007177771	
Ural		**6(4.0)**		
Ural-1	777	4(2.6)	777777777420771	
Ural-1	Orphan	2(1.3)	777777747420771	
Not defined	New	1(0.7)	777777770000771	
Not defined	New	1(0.7)	700000017774771	
Not defined	New	1(0.7)	777774777420771	
Not defined	New	2(1.3)	770000037760771	
Latin American Mediterranean		**5(3.3)**		
LAM9	1176	2(1.3)	777757607760771	
LAM9	42	3(2.0)	777777607760771	
East African Indian		**1(0.7)**		

EAI5	126	1(0.7)	477777777413771	
Central Asia Strain		29(19.2)		
CAS1-Delhi	22	7(4.6)	703777400001771	
CAS1-Delhi	25	2(1.3)	703777740003171	
CAS1-Delhi	26	14(9.3)	703777740003771	
CAS1-Delhi	289	3(2.0)	703777740003571	
CAS1-Delhi	357	1(0.7)	703777740000771	
CAS1-Delhi	429	1(0.7)	703777740003731	
CAS1-Delhi	794	1(0.7)	703757740003771	

7. Discussion

This study aimed to determine the molecular characterization and drug resistance patterns among EPTB in Addis Ababa, Of 151 isolates, twenty-two (14.6%) of isolates were resistant to INH and similar numbers were resistant to PZA. The overall proportion of MDR-TB was 9.3%. Of 14 MDR-TB isolates, 21.4% were developed pre-XDR-TB. A rpoB mutation indicates that 15 (9.9%) were resistant to RIF. Eighteen (11.9%) had amino acid mutations at the S315T1 position. Three (2.0%) *M. tuberculosis* isolates had discordant results between the genotypic MTBDR*plus* test and the phenotypic DST. We have identified three (2.0%) *M.bovis.* The most predominant lineages were also T (55.0%), Central Asia Strain (19.2%), and Haarlem (8.0%). The most predominant strain types were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%).

We have also investigated demographic, clinical and microbial characteristics of 151 EPTB isolates. In this study, the proportion of lymph node TB was the most common site (65.6%) which followed by pleural TB (21.1%). Also, most of EPTB were associated with specific young-age adult groups from 15 to 39 years. This observation is relatively similar to the findings of previous studies in Ethiopia [23] and India [25]. In our study, male sex was slightly more affected by EPTB than females (54.3% vs. 45.7%). This observation is consistent with the findings of previous studies reported in northern Ethiopia [32], Mozambique [30], and Turkey [28]. This might be due to under-diagnosis of TB in females as a result of various social and/or cultural factors, including their consequent impaired access to health care.

In this study, we were performed liquid DST for STM, INH, RIF, EMB and PZA. Also, second-line drug sensitivity testing was performed for AMK, CAP, ETH, KAN, MOX, and OFX for all MDR-TB isolates. In this study resistance against INH (14.6%) was the most frequent. This proportion is to some extent higher than an earlier report in Ethiopia [23] and Thailand [46]. However, our finding is similar to the results reported from India (13.3%) [47], and South Korea (12.8%) [49]. Moreover, two studies reported from India indicated higher (more than 30%) resistance against INH [48, 50] than our finding in which only 14.6% were resistant to INH. This difference could be due to the study area, sample size. Unlike our study conducted on a statistical optimum sample size the two studies reported from India were conducted on small sample size.

The most frequent resistance proportion was observed against PZA (14.6%). However, since there is no sufficient data on PZA resistance of EPTB globally, we could not compare it with the previous study. This study could serve as a reference for further study on resistance against PZA in EPTB patients. Although the proportion of PZA resistance is high in *MTB* cases, the phenotypic PZA susceptibility test is rarely performed due to technical difficulties [51]. PZA is active only at low pH which requires acidic culture medium for susceptibility testing. The acidic nature of the culture medium prevents about 20 to 25% of isolates from growing [51]. On the other hand, a large inoculum size also can cause alkalization of the medium that leads to false PZA resistance [51]. Due to this technical challenge in the laboratory and the possibility of false resistance most TB laboratories do not perform PZA susceptibility testing.

In our study, 9.3% of isolates were tested resistant against RIF which is in agreement with the previous study reported India in which 10.1% resistant against RIF [47]. However, higher resistance against RIF was reported from Ethiopia and India [23, 48, 50]. Relatively lower RIF resistance proportion was reported from India and Thailand [47, 46]. This variation in resistance against RIF might be due to geography and the use of different sample sizes.

In our study, 9.3 % of isolates were diagnosed with MDR-TB. Among all MDR-TB cases, 21.14 % were from previously treated patients and 8.0 % were from newly diagnosed cases. Previous studies reported from India indicated relatively higher MDR-TB cases [48, 47, 50] than our findings. In contrast, two studies from South Korea [49, 52] and one study from Thailand [47] are reported lower MDR-TB proportion than our findings. The possible reason for high-level drug resistance in our findings than the previous studies results is due to non-adherence, loss to follow up, poor drug supply chain management, and poor quality of drugs [56]. Moreover, high MDR-TB in our study might be due to that the study was conducted among the population of TB or MDR-TB suspected patients included in the studies. However, our finding is relatively consistent with the findings of previous studies reported from India, China, and South Korea [50, 57, 54, 55, 49].

The result of high MDR-TB is one of the growing problems which have led to an increase in other drug-resistant cases of TB such as pre-XDR-TB and XDR-TB cases. For this reason, MDR-TB cases should be timely monitored. The proportion of pre-XDR-TB cases among MDR-TB patients in this study was 20%. A similar study was low reported Fluoroquinolones resistance in

Pakistan the rate of 6.0% [53]. The findings of this study are relatively similar to studies reported from India in which the proportions of pre-XDR-TB among MDR-TB patients were 18.4% [57]. On the other hand, the study reported from India indicated a higher proportion (38.2%) of pre-XDR-TB among MDR-TB patients [58]. In our study, the higher (20%) pre-XDR-TB was resistance against FLQ than injectable second-line drug-resistant. A study reported from India indicated a higher proportion (38.2%) of resistance against FLQ [58] than our finding of pre-XDR-TB. In contrast, another study reported from India indicated a lower proportion (3.4 %) of pre-XDR-TB that resistance to FLQ [47] than our study finding. The reason for the higher number of pre-XDR-TB cases in MDR-TB found in this study might be due to treatment failure that intensified the population drug-resistant strains [58]. Moreover, the possible explanation might be antibiotics are previously used for treating pneumonia which increases the resistance against these drugs. For example, fluoroquinolones and other drugs are broad-spectrum antimicrobial agents that widely and indiscriminate used particularly at sub-therapeutic doses. The wide use of these drugs most likely leads to an increase in quinolone-resistant. Thus, the indiscriminate use of these antibiotics might be contributed to the high proportion of pre-XDR-TB cases in this study [60].

Understanding the nature and frequency of mutations associated with drug resistance in *MTB* is important for the development and genetics-based assays for the diagnosis of drug resistance [61]. Early detection of drug resistance mutations would assist TB patient management and avoid treating individuals with inefficacious regimens [62]. The MTBDR*plus* and MTBDR*sl* assay have been widely used in clinical routines for the identification of MTB complex and detection of first and second-line drug resistance due to its shorter turnaround time. The direct use of the assay on clinical specimens is another key advantage, as this prevents waiting for cultures to grow.

Rifampicin resistance is related to mutations in a restricted area of the rpoB gene. In the Genotype MTDR*plus* test, Rifampicin resistance is related to the absence of one or more bands WT (WT1 to WT8) or the presence of the mutation band (MUT1, MUT2A, MUT2B, and MUT3) in area 500-531 of the rpoB gene [63]. In the present study, the most frequently observed mutations on the gene rpoB were the absence of bands WT8 (8.6%) and WT7 (1.3%). The presence of mutation on S531L and H526Y was lower than the previous studies reported from Iran and India [64 65 66]. However, our study finding was similar to the previous study reported from Sudan on S531L and H526Y mutations [67]. All RIF resistant isolates in this study were reported to have their

mutations rifampicin resistance determining region of rpoB codons 516 to 533. This finding is in agreement with a study reported in Iran [64].

The higher frequency of resistance to INH occurred due to mutation of the katG gene, whereas the lower frequency of resistance was caused by the mutations in the promoter region of the inhA gene [68]. In our study, from all INH resistant strains, 11.9% of strains had S315T1 mutation in katG region that led amino acid serine substitution to threonine. This was smaller than the result found in Northern India where 94.5% of isoniazid resistance isolates had a mutation in the katG gene [65]. Still studies reported from India also indicated mutation in codons S315T1 of katG 100% association with INH resistance [61, 66]. Moreover, the study reported from Sudan indicated that S315T1 mutation and mutation in the inhA gene that occurred in C15T are associated with INH resistance [67]. However, the same study reported in India is shown a higher proportion of C15T mutation [65]. Similarly, a study reported from Sudan found C15T mutation that resulted in INH resistance [67]. These findings are in agreement with our results in which mutation of C15T associated with INH resistance

As the burden of XDR-TB is increasing in MDR-TB patients, there is an urgent need for rapid and accurate tests for XDR-TB. XDR-TB is caused by strains of *MTB* that are resistant to INH, RIF, and any of the FLQs and at least one second-line injectable agent [69]. In our study, we found 20% of FLQ resistance among the 15 MDR-TB or RR-TB strains. Our finding is similar to the studies reported from Bangladesh [70] and India [57] in which FLQ resistance proportions were 19.11% and 18.4% respectively. The finding of our study is also corresponding with the global FLQ resistance proportion which is 17% among MDR-TB strains in 2014 [71]. Also, another study reported from New Delhi, India indicated a similar proportion (17.1%) of FLQ resistance among MDR-TB patients with our results (20%). Moreover, hospital-based studies reported from Wuhan, China found 22% of OFX resistance in TB suspects [72, 73]. In our study mutation in codon at gyrA was detected in 20% of FLQ resistant isolates. This mutation confers resistance to levofloxacin and is associated with low-level resistance to moxifloxacin [74]. In the present study, gyrA mutations were predominantly found to occur in codons 89–93, 92–96, S91P, and A90V. These most common mutations are largely corroborating the findings of the previous study [75]. The predominant rrs gene mutation was A1401G (6.6%). This is a common mutation reported in several studies to be associated with high-level resistance to AMK, KAN, and CAP

[76, 77]. In our study the absence of A1401G mutation was found in the gyrB and eis gene among MDR strains. The mutations in the gyrB gene are usually associated with low-level resistance to fluoroquinolones and are not as common as those in the gyrA gene [78]. This could be due to the limited number of resistant isolates to second-line drugs.

In the current study, we compared the performance of the line probe assay with the MGIT 960 system for the detection of drug susceptibility tests. In our study, DST results by BACTEC MGIT 960 were used as the 'gold standard' as this method has detected resistance of M. tuberculosis isolates to all drugs accurately. On two (1.3%) isolates discordance between molecular and phenotypic methods in drug susceptibility was confirmed for INH. Of these isolates, phenotypic DST has revealed resistance to INH, while genotypic provided susceptible results. Discordant results between phenotypic DSTs and genotypic DST may not be due to all mutations conferring resistance to anti-TB drugs being included in LPA assay. Resistant mutations may result in variable have low, moderate, or high phenotypic expression of drug resistance to INH. Silent mutations do occur at the genetic level with no change in drug susceptibility patterns. The LPA assay may miss silent or neutral mutations that result in phenotypical susceptibility. This could have been due to unidentified mutation in some other genomic region (like ahpC, kasA, furA) which is not targeted by this assay [79].

One (0.7%) isolate was shown resistance to RIF in the genotyping DST, but susceptible in phenotyping DST. The discordant results of DSTs for RIF between the genotypic MTBDR*plus* test and the phenotypic test result is our finding is similar to findings reported from Bangladesh and Congo Kinshasa [80, 81]. This could be due to the presence of mutations outside of the rpoB hotspot region to the target region for MTBDR*plus* assay. Another resistance mechanism such as hetero-resistance through mixed patterns of WT and mutant melt probe might be present. This could be because of some rare mutations occurring outside the 81bp region of the rpoB gene which is not targeted in our assay [79].

In this study, we compared the sensitivity and specificity of the line probe assay with the MGIT 960 system for the detection of drug susceptibility to first-line drugs. Line probe assays showed high sensitivity and specificity of the detection of susceptibility to RIF (sensitivity 100% and specificity 99.3%) and INH (sensitivity 90.9% and specificity 100%). In this study, relatively low sensitivity and specificity were observed in India for the detection of RIF (sensitivity 92.7% and

specificity 99.3%) and (sensitivity 95.2 and specificity 100%) [82, 83]. The specificity of 99.3% in the case of rifampicin resistance is in agreement with the previous reported was 97.30% [66].

Sensitivity and specificity of 99.3% and 100% for the detection of INH resistance are lower with the studies in India where a sensitivity of 93% and specificity of 97% were seen [77]. The sensitivity of the GenoType MTBDR*plus* assay for the detection of INH resistance in the present study was lower than those reported by previous studies in Ethiopia [84, 85] Uganda [86] and Pakistan [87]. The MTBDR*plus* assay was not able to detect the INH resistance in the two isolates, which were detected by the MGIT 960 system. This could be due to mutations in the inhA promoter and the ahpC-oxyR intergenic regions, which have not been included in the strips [88] or it could also be due to unidentified mutation. The high specificity of the MTBDR*plus* assay in detecting INH resistance and RIF resistance isolates agrees with the specificity of previous studies [84-86]. RIF resistance in one isolate was detected by the MTBDR*plus* assay but not by MGIT 960 system. Being susceptible by phenotypic method but resistant by the genotypic method may be associated with false RIF resistance due to a silent mutation which makes the probe fails to hybridize on a strip and interpreted as RIF resistant [89]. Recent studies have shown that these uncommon gene mutations may be susceptible or have low-/high-level resistance to isoniazid. We suggest the WHO recommends that DSTs of both phenotypes and genotypes should be used for all DST of first-line anti-TB drugs for diagnosis [90, 91].

In this study, molecular characterization of the strains of *M.tuberculosis* using spoligotyping identified 41 different spoligotype patterns from 151 isolates. Out of 151 isolates, 146(96.7%) patterns have matched a preexisting shared type in the SITVIT2 database, whereas the remaining 5(3.3%) patterns were not found in the SITVIT2 database. Our results of 151 MTBC isolates was shown a high diversity of strains such as Beijing, X1, Turkey, Haarlem, Bovis, T, Ethiopia, Ural, Central Asia Strain, East African Indian, and Latin American Mediterranean. There were also some orphan strains assigned to their most appropriate lineage and sub-lineage using the TB insight database. Most of the lineage highly prevailed in the current study were T (55.0%), CAS (19.2%), and H (8.0%). The most predominant strain spoligotypes were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%). The occurrence and distribution of these families vary from region to region in Ethiopia. These results are compatible with two earlier studies reported from Ethiopia [27, 33]. The study reported from central Ethiopia indicated a high proportion of SIT53 and

SIT149 strains [33, 92]. Moreover, a study reported from Addis Ababa shown a high proportion of SIT53 strain and CAS family strains [27]. The number of SIT149 isolates in our study was thrifty two isolates higher the number registered in the SITVIT2 international database. This might be due to the association of this familiar strain with East African countries, especially in Ethiopia. Also, the presence of genetic diversity in the present study could be since the strains may have entered by the traders and other activates from the different regions as the study included participants from a town nearby Addis Ababa.

In our study, 2.0% of *M.bovis* was identified by GenoType MTBC Assay and Spoligotype pattern. The previous study has detected *M.bovis* in extrapulmonary TB [30] which is similar to our finding. Moreover, similar findings were reported previously from Ethiopia in which the proportion of *M. bovis* is 1.7% [23]. Besides, the study reported from India on extrapulmonary tuberculosis patients indicated 1.5% of the isolates had *M. bovis* [26]. The low proportion of *M.bovis* in this study could be attributed to the fact that participant's occupational background in this study was not associated with livestock or farms. This is because *M. bovis* strain is spread to a human through contact between to infected domestic animals such as cattle, wild animals, and ingestion of unpasteurized milk or contaminated meat. Also, the second usual route of infection with *M. bovis* is through animals and humans inhaling infected droplets that are expelled from the lungs by coughing.

Our study identified five isolates are new spoligotype patterns of *Mycobacterium tuberculosis* which were not present in the international database. This indicates that there is little information about *MTB* strains circulating in the study area. Since only a small number of isolates were characterized in our study, it is not possible to appreciate the diversity of *MTB* circulating in the study area. More isolates and wider geographical coverage are required to have a detailed insight about the *MTB* strains circulating in Addis Ababa.

8. Strength and limitation of the study

8.1. Strength

The strengths of this study were including different types of clinical specimens for EPTB patients and among the few studies that reported drug resistance and molecular characterization of EPTB in Ethiopia.

8.2. Limitation

The main limitation of this study was the lack of detailed patients' characteristics to identify risk factors associated with drug resistance in patients infected with EPTB. Also, the being participants were from a single city limited to the generalizability of this study finding at the national level. However, despite the limitations stated above, we do believe that the results of this study were less likely to be influenced by those limitations.

9. Conclusion and Recommendation

9.1. Conclusion

The present study provides an overview of molecular characterization and drug resistance *MTB* from extrapulmonary patients in Addis Ababa. In this study about three-fourth of the isolates shown T and Central Asia strain lineages. A high proportion of INH and PZA resistance, MDR and Pre-XDR were identified in EPTB isolates. The most mutations associated with first-line drug resistance occurred at catG gene (codon S315T1) region, while second-line drug resistance occurred at gyrA gene (codons 92–96).

9.2. Recommendations

The number of isolates obtained for this study was small and sample collection was limited to one city, Addis Ababa, which is limited to the generalizability of our results to the national level. Thus, large scale future study on molecular characterization and drug resistance of *MTB* in EPTB is required. Furthermore, the use of more accurate molecular techniques that could discriminate the mutation regions is recommended. High drug resistance proportion detected in this study also demands against immediate action to prevent the further occurrence of drug resistance. Resistance to PZA and INH was high indicating further investigations is required to know the exact cause and the possible contribution of isoniazid preventive therapy.

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10. Annexes

Annex 1 Protocol for Phenotypic DST

Purpose

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

Principle

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

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

Procedure BACTEC MGIT 960 culture

1. Label BACTEC MGIT 960 tubes with isolate number.

2. Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA
3. Add up to 0.5 ml of a well-mixed isolate to the appropriately labeled MGIT tube.
4. Incubate MGIT tube entered in the BACTEC MGIT 960 instrument after scanning each tube
5. Select Positive isolates for SD Bioline TB Ag MPT64 Rapid test SD Bioline TB Ag MPT64 Rapid test
6. Label each cartridge with the sample identification number
7. Place 100µL of the prepared bacterial culture on the specimen placing area of the test cartridge. Pipette tips should be changed between samples
8. A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Line
9. The formation of a purple to red line on the reading areas labeled [T] and [C] of the cartridge indicates a POSITIVE result
10. The right section of the result window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Line
11. The formation of a purple to red line on the reading area labeled [C] of the cartridge but not [T] indicates a NEGATIVE result
12. If no line is observed on the reading area [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new cartridge 13. Examine the reading area of the test plate after 15 minutes

Drug Susceptibility test using BACTEC MIGT 960

1. Select a positive isolates and ready for DST (For the preparation of the test inoculum, a positive 7 mL MGIT tube should be used the day after it first becomes positive on the BACTEC MGIT 960 instrument (Day 1), up to and including the fifth day (Day 5) after instrument positivity.
2. The tube is a Day 1 or Day 2 positive, mix well and proceed to “Inoculation Procedure for Susceptibility Test.” If the tube is a Day 3, Day 4, or Day 5 positive, mix well then dilute 1 mL of positive broth in 4 mL of sterile saline (1:5 dilution). Use the diluted suspension for the inoculation procedures.
3. Label each MGIT tube with relevant drug and laboratory number and date

4. Reconstituting lyophilized antibiotics in distilled water and MGIT tubes supplemented with 0.8 ml of the enrichment solution (BactecMGIT SIRE supplement; Becton Dickinson), the critical concentration for each drug was 5.0µg/ml, 0.1µg/ml, 1.0µg/ml and 1.0µg/ml for ethambutol, isoniazid, rifampicin and streptomycin respectively.
5. Add appropriate reconstituted drug solutions into each of the corresponding labelled BACTEC MGIT 960 tubes.
6. Aseptically pipet 0.1 mL of the organism suspension into 10 mL of sterile saline to prepare the 1:100 Growth Control suspensions for day 1 and day 2 isolate. Mix the Growth Control suspension thoroughly.
7. Aseptically pipet 0.5 mL of the organism suspension from the original isolate into each of the FOUR remaining drug tubes (STR, INH, RIF,EMB).
8. Inoculate 0.5 ml of this suspension into the growth control-labelled tube from 1:100 diluted tube
9. Immediately recap the tube tightly and mix by inverting the tube several times.
10. Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
11. Be sure that the tubes are loaded according to the order specified for the AST set entry feature. Be sure that the caps are tightly closed
12. Open the desired MGIT 960 drawer and press the “tube enter” key.
13. The barcode scanner will light up
14. Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
15. Incubate MGIT tubes until the instrument flags them as positive
16. Check MGIT 960 daily for indicator lights flagging positive
17. Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer

Note: Second line drug susceptibility test using BACTEC MIGT 960 are the same procedure except drug and drug concentration different.

Annex 2: Protocol Genotypic DST or Line probe assay

Purpose

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

Principles

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

Amplification: Before amplification, amplification Mixes A (Taq polymerase, PCR buffer and nucleotides) and B(biotinylated primers, Mgcl 2 and are optimized for the PCR step of MTBDRplus test. The nucleotides acts as DNA precursors which will be used as building blocks during DNA polymerase (Hot Start Taq) elongate the DNA molecule.

Reveres Hybridization:

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

Procedures

Extraction:

1. Using a sterile disposable Pasteur pipette, transfer 1000µl of each thoroughly mixed liquid culture sample to labelled 1.5ml screw cap tube.
2. Proceed similarly to the procedure for decontaminated smear positive sediments
3. Load the 1.5ml screw cap tubes in a micro-centrifuge with aerosol-tight rotor.
4. Centrifuge for 15 minutes at 10,000 RCF or 10263RPM
5. Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC
6. Discard supernatant from each tube by use of a 1000µl adjustable pipette
7. Resuspend each pellet in 100µl Lysis Buffer (A-LYS)

8. Mix the contents of each tube by use of a sterile tip followedby thorough vortexing for at least 15 to 20 seconds
9. Incubate the tubes for 5 minutes at 95 °C in a thermoblock
10. Add 100 µl Neutralisation Buffer (A-NB) and vortex the samplefor 5 seconds
11. Load the tubes into a microcentrifuge and spin for 5 minutesat 13,000 x g
12. Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube
13. Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °C

Amplification and Detection:

1. Prepare a master mix containing AM-A and AM-B in a sterile screw cap tube (1.5ml) according to the number of samples and controls
2. Pipette 45 µl of the master mix to each labelled PCR tube
3. Add 5µl of DNA to corresponding master-mix PCR tubes in the PCR hood.
4. For first use, set up the thermal cycler to the correct amplification profiles according to the instruction manual.
5. Run the specific program (Ver.2-cul for samples from solid or liquid culture)
6. After the cycles are complete, proceed to the detection stage. If detection cannot be performed on same day, store PCR tubes with amplicons at 4°C for a maximum of 7days
7. Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total). Pre-warm RIN (rinse solution) and sterile distilled water to room temperature
8. Pre-warm TwinCubator to 45°C
9. Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided pen according to detection worksheet.
10. Pipette 20µl DEN (denaturing solution) to one end of each well of a clean tray to be used
11. Add 20µl of corresponding amplified DNA sample to the denaturing solution in each well, and mix well by pipetting up and down 5 times
12. Incubate for 5 minutes at room temperature

13. Carefully add 1 ml HYB (hybridization solution) to each well and in the opposite end to the DEN/DNA mixture. Use single tip for each well. Do not splash mixture or contaminate neighbouring well
14. Gently tilt to shake and homogenize solution. Do not splash mixtures
15. Add each labelled strip to each well with coloured marker facing up. If strips turn over, re-position them with a fresh pipette tip. Strips must be completely covered by hybridization solution
16. Place tray on Twincubator and press "START" to incubate for 30 minutes at 45°C. From this point, press right arrow on Twincubator once to advance steps in protocol.
17. When alarm goes off, press right arrow key to stop
18. Remove HYB carefully by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change tips or Pasteur pipettes between wells
19. Wipe off condensation that forms on Twincubator lid before every incubation step.
20. Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in Twincubator at 45°C. Press right arrow key to start.
21. When alarm goes off, press right arrow key. Completely remove STR as previously described for HYB removal.
22. Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips for 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.
23. Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on Twincubator
24. When alarm goes off, press right arrow to stop
25. Completely aspirate CON-D solution using Pasteur pipette
26. Add 1ml RIN per well. Press right arrow and incubate for 1 minute on Twincubator
27. When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20
28. Remove RIN completelyand wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator
29. When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.

30. Place on Twincubator under aluminum foil for a maximum of 10 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes
31. Wash twice for 1 minute with distilled water. Remove distilled water after each wash
32. Use forceps to transfer membrane strips to an absorbent paper and allow to air dry
33. Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water
34. Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol
35. Switch off the Twincubator after use
36. Use forceps to transfer strips to the GenoType MTBDRplus Results Sheet provided with the kit
37. Align the bands Conjugate Control (CC) and Amplification Control (AC) on each strip with the respective lines on the sheet
38. Attach the strips to the results sheet using clear adhesive tape
39. Determine the band positivity and positions on each strip using the reference reading chart of the kit and mark the results on the worksheet
40. In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.
41. If a positive result is obtained with the negative control, the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
42. In order for patient results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that M.tuberculosis complex is present in the sample
43. If CC is negative the conjugation or substrate reaction was unsuccessful either due to error in the procedure or due to problems with the reagent
44. If AC is positive, errors during extraction and amplification set-up and presence of amplification inhibitors in the specimen can be excluded
45. Signal of AC can be weak or even absent while results for other bands (TUB, rpoB, katG and inhA locus controls) may be positive. This might be due to competitive reactions

between AC and TUB, ropB, katG, inhA during amplification. In this case, the strip can be evaluated.

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

Note: Second line LPA test using method are the same procedure except master mix containing AM-A, AM-B and DNA strips different from first line LPA methods

Annex 3: Protocol for Genotype MTBC

Purpose

The GenoType MTBC test is a qualitative in vitro test from cultured material for the differentiation of the following species/strains belonging to the Mycobacterium tuberculosis complex (MTBC): M. tuberculosis/M. canettii, M. africanum, M. microti, M. bovis subsp. bovis, M. bovis subsp. caprae, and M. bovis BCG.

Principles of the Procedure

The GenoType MTBC test is based on the DNA•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from cultured material (solid/liquid medium; the necessary reagents are not provided), (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization. All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus the probes reliably discriminate the different sequences of the bacterial species. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

DNA Extraction

Bacteria grown on culture plates (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. BACTEC, MB-Check) may be used as starting materiafor DNA extraction. The working area must be free from contaminating DNA.

The following quick protocol can be used for DNA extraction from cultured material:

1a. When using bacteria grown on solid medium, collect bacteria with an inoculation loop and suspend in approximately 300 µl of water (molecular biology grade).

1b. When using bacteria grown in liquid media, directly apply 1 ml. Pellet bacteria by spinning for 15 min in a standard table top centrifuge with an aerosol tight rotor in a class II safety cabinet at approximately 10,000 x g. Discard supernatant and resuspend bacteria in 100-300 µl of water (molecular biology grade) by vortexing.

2. Incubate bacteria from 1a or 1b for 20 min at 95°C in a water bath.

3. Incubate for 15 min in an ultrasonic bath.

4. Spin down for 5 min at full speed and directly use 5 µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, transfer supernatant to a new tube.

The assay on hand was also validated with the GenoLyse® kit (see chapter Ordering Information) which can alternatively be used for DNA extraction. For handling instructions, please refer to the instructions for use of the GenoLyse® kit.

The methods described above were used for performance evaluation of the GenoType MTBC. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.

Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. After thawing, stir AM-A and AM-B carefully. Pipette AM-A and AM-B only in a room free from contaminating DNA. The DNA sample should be added in a separate area.

Prepare for each sample:

- 10 µl AM-A (see Kit Component 2)
- 35 µl AM-B (see Kit Component 2)
- 5 µl DNA solution
- Final volume: 50 µl

Hybridization

When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the

hybridization protocol to be used. The following protocol describes the manual hybridization using a water bath or a TwinCubator.

Preparation

Prewarm shaking water bath to 45°C (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on TwinCubator. Rewarms solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1. Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.
2. Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
3. Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color. Take care not to spill solution into the neighboring wells.
4. Place a strip in each well. The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.
5. Place tray in shaking water bath/TwinCubator and incubate for 30 minutes at 45°C. Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.

6. Completely aspirate Hybridization Buffer. For example, use a Pasteur pipette connected to a vacuum pump.
7. Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator.
8. Work at room temperature from this step forward.
9. Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).
10. Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator.
11. Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water
12. Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.
13. Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.
14. Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is provided with the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and UC with the respective lines on the sheet. Note down positive signals in the last but one column, determine species with the help of the interpretation chart and enter name of the identified species in the last column. The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and UC of the strip as well. Each strip has a total of 13 reaction zones

Troubleshooting

Overall weak or no signals (including Conjugate Control zone)

- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used. Repeat reverse hybridization.

Weak or no signals except for Conjugate Control zone

- Quality of extracted DNA does not allow an efficient amplification. Repeat extraction.
- Amplification Mixes (AM-A and AM-B) were interchanged, added in wrong amounts, or not stirred properly. Prepare a new master mix and repeat test.
- Incubation temperature too high. Repeat reverse hybridization.
- The extracted bacterial species cannot be detected by the Universal Control. Use alternative identification method.

No homogeneous staining

- Strips were not completely immersed during incubation steps.
- Tray was not shaken properly. Repeat reverse hybridization.

High background color

- CON-C and/or SUB-C used too concentrated.
- Washing steps were not performed with the necessary care.
- Wash solutions too cold. Repeat reverse hybridization.

Unexpected result

- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer. Repeat reverse hybridization.
- Contamination of extracted DNA with previously extracted and/or amplified DNA. Repeat extraction.
- Contamination of amplification reagents. In this case, a negative control sample shows additional bands besides CC. Repeat amplification using fresh reagents.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands.
- No pure culture as starting material. Re-culture in order to exclude contamination.

- Error during DNA extraction. Repeat extraction.
- The bacterial species present in the sample cannot be detected with this test. Apply additional detection methods.

Annex 4: Protocol for Spoligotyping

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

Spoligotyping Principle

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

Procedure

1. Always include chromosomal DNA of Mycobacterium tuberculosis strain H37Rv and Mycobacterium bovis as positive controls and primary buffer use as a negative control.

A. **Prepare the Master Mix reaction:**

Reagent	1	50
HotStarTaq Master Mix	12.5ul	625
Primer (DRa)	2ul	100
Primer (DRb)	2ul	100
Molecular grade Water	3.5ul	175

a. Add 5ul of your sample
b. Select the program from the PCR machine
c. Make sure the volume is 25ul
d. Run the PCR
e. Store at 2-8 ^{O}C till detection

Reagent preparation

Reagent used for hybridization

Primary buffer (2XSSPE/ 0.1SDS)

250ml/round=25ml 20XSSPE + 222.5ml distill water + 2.5ml 10%SDS

- Used to dilute PCR product –allocate 10ml and store at room temperature
- Used to pre-warm the membrane-the remaining 240ml will be equilibrate at **60^{O}C**

Secondary buffer (2XSSPE/ 0.5SDS)

1000ml/round=100ml 20XSSPE + 850ml distill water + 50ml 10%SDS

- Used to remove unbound PCR product and residual POD
- Split the buffer into two: Equilibrate 500ml at **60^{O}C** and 500ml at **42 ^{O}C**

2XSSPE

500ml/round= 50ml 20XSSPE + 450ml distill water

- Used to remove excess SDS: store at **room temperature**

1. Reagent used for membrane washing and storage

1%SDS

500ml/round= 50ml 10%SDS + 450ml distill water

- Used to remove the PCR product: Equilibrate at **60^{O}C**

20mM EDTA

500ml/round= 20ml of 0.5EDTA + 480ml of distill water

- Used to wash and store the membrane: Store at room temperature

Hybridization with PCR product and detection

1. All buffers should be pre-warmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):
   - Primary buffer (2×SSPE/0.5 % SDS, at 60 °C)
   - Primary buffer (2×SSPE/0.5 % SDS, at 42 °C)
   - Seconder buffer (2×SSPE/0.1 % SDS, at 42 °C)

- 2×SSPE, room temperature.

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

Detection procedure

1. Prepare ECL by adding 10ml of solution 1 and 10ml of solution 2
2. Use specific container marked as “ECL” and add the ECL solution to the membrane
3. Immerse for 1-2min and ensure the membrane is completely covered in the solution
4. Use cassette and prepare used film rapped with plastic (to support the membrane) and place the membrane on it
5. Cover with plastic sheet and avoid bubble
6. **Work in dark room**, put a new film on top, close the cassette, and expose for 10-20min (depending on the intensity of black square)
7. Ensure film developer (100ml), water and fixer(100ml) is ready
8. Turn on the red light, remove the film and place into the developer. Ensure it is entirely submerged

9. Tilt gently utile an image of black square is visible
10. Rinse with distilled water for 5-10sec
11. Immerse into trays containing fixer, ensure it is entirely submerged. Agitate gently for 1-2min
12. Rinse with water for at least 2min and allow to dry
13. Label the film with round number and DRS number
14. Discard the developer but transfer the fixer into labeled dark bottle. The fixer can be used for 5 times (with maximum one month)

Trouble shooting

1. Spoligotyping demands that the procedures are followed in an accurate and careful way. This improve our final result
2. However, the list below enhance our result in case of poor spoligopattern
3. In case of weak spoligopattern, the signal can be enhanced by longer the exposure time of the film to the membrane i.e. 30-40min
4. In case of strong spoligopattern, the signal can be improved by shorter the exposure time of the film to the membrane i.e. 10min
5. In case of patchy or dark shadows spoligopattern, wash the membrane **two times** in 250ml of 2XSSPE at room temperature for 5min with shaking and repeat the whole detection procedure

A. Regeneration of the membrane (Membrane washing procedure)

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

1. Unwrap the membrane and wash **two times** with 250ml of 1% SDS (stored at 60 ^{O}C) for 30min at 80 ^{O}C with shaking
2. After the second wash leave at room temperature for 5min to cool before discarding the liquid
3. Wash for 5min in 250ml of 20mM EDTA
4. Discard the liquid & add another 250ml of 20mM EDTA & store the membrane at 2-8 ^{O}C.

Declaration

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

M.Sc. candidate: Getu Diriba (B.Sc.)

Signature_____

Date of submission_____

This thesis has been submitted with our approval as advisers.

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ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Molecular characterization and drug resistance pattern of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia

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A research thesis submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, in partial fulfillment of Master of Sciences Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

June 2020

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Getu Diriba, entitled: "**Molecular characterization and drug resistance pattern of *Mycobacterium tuberculosis* complex among confirmed extra pulmonary tuberculosis cases in Addis Ababa, Ethiopia**" submitted in partial fulfillment of the requirements for the degree of Master of Science in Clinical Laboratory Scinces (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Approved by the Examining Committe:

External Examiner _____ Signature_____ Date_____

Internal Examiner _____ Signature_____ Date_____

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_____

Chairperson of the Department of Graduate program Coordinator

Acknowledgment

I would like to express my deepest gratitude to my advisors Mr. Kassu Desta, Mr. Dessie Abera, Mr. Abebaw Kebede, and Dr. Habteyes Hailu for their guidance during the implementation of the study your scientific advice, support and encouragement gave me the confidence to think critically and work independently to improving the quality of this thesis. I also acknowledge Addis Ababa University, College of Health Sciences, Department of Medical Laboratory Sciences for financial support, and Ethiopian Public Health Institute (EPHI) National Tuberculosis Reference Laboratory (NTRL) for their willingness to support this study by providing laboratory chemicals, reagents, and permission to use laboratory facilities. I would like to express my gratitude to Mr. Bazezew Yenew, Mr. Gebeyehu Asefa, and Mrs. Muluwork Getahun for their boundless support during drug sensitivity testing and spoligotyping analysis. I would also extend my gratitude to Mr. Zemedu Mohhamed for his support on data analysis. I am also thankful to all staff members of EPHI NTRL for their support and fun during the laboratory work of this thesis project.

Lastly, but not least, I would like to acknowledge colleagues in the graduate students of Addis Ababa University, Department of Medical Laboratory Sciences for their support. I would express my gratitude to my family, my wife and all friends who have contributed directly or indirectly to this work and encouraged me.

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List of Abbreviations

AAU	Addis Ababa University
AFB	Acid-fast bacilli
AMK	Amikacin
BCG	Bacille Calmette–Guerin
CAP	Capreomycin
CAS	Central Asia Strain
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
EA	Euro-American
EAI	East-African Indian
EMB	Ethambutol
EPHI	Ethiopian Public Health Institute
EPTB	Extrapulmonary Tuberculosis
ETH	Ethionamide
FNAC	Fine Needle Aspirate Cytology
FQs	Fluoroquinolones
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IO	Indio Oceanic
KAN	Kanamycin
LAM	Latin-American Mediterranean
LAMP	Loop-mediated isothermal amplification
LPA	Line Probe Assay
LTBI	Latent Pulmonary Tuberculosis Infection
MDR-TB	Multidrug Resistance Tuberculosis
MGIT	Mycobacterium Growth Indicator Tube
MOX	Moxifloxacin
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MUT	Mutant

NAA	Nucleic Acid Amplification
NAAT	Nucleic Acid Amplification Test
NTM	Nontuberculous *Mycobacteria*
NTRL	National Tuberculosis Reference Laboratory
OFX	Ofloxacin
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
RIF	Rifampicin
RR	Rifampicin Resistance
SLD	Second-line Drugs
SIT	Spoligotype International Type
SIRE	Streptomycin Isoniazid Rifampicin Ethambutol
SPSS	Statistical Package for Social Scientist
SSPE	Saline-Sodium Phosphate EDTA
STM	Streptomycin
TB	Tuberculosis
VIO	Viomycin
WHO	World Health Organization
WT	Wild type
ZN	Ziehl-Neelsen

Abstract

Background: Molecular characterization and drug-resistant of *Mycobacterium tuberculosis* is a valuable tool in understanding the pathogenesis, diagnosis treatment, and prevention of tuberculosis. However, there is limited information on molecular characterization, and drug-resistant patterns of *Mycobacterium tuberculosis* in patients with extrapulmonary tuberculosis in Ethiopia.

Objective: This study aimed to determine the molecular characteristic and drug resistance patterns of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia

Methods: A laboratory-based cross-sectional study was conducted among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia in a period between November 2019 and April 2020. Drug susceptibility test was performed using BACTEC-MGIT 960 and Line probe assay. Strain identification was performed using the Geno-Type MTBC and spoligotyping technique. Data were entered into the international spoligotyping database to assess the spoligotype patterns of *Mycobacterium tuberculosis*. Data was analyzed by SPSS version 23 and participants' characteristics were presented by number and proportion.

Results: Of 151 *Mycobacterium tuberculosis* complex isolates, 29 (19.2%) were resistant to at least one drug, and 14 (9.3%) were identified as multidrug-resistant tuberculosis. Besides, 21.4% (3/14) of isolates had pre-extensively drug-resistant tuberculosis. S315Tl (11.9% (18/151) was the most common isoniazid mutation observed in the katG gene. The spoligotyping of the 151 isolates resulted in 41 different spoligotype patterns. The most abundantly represented SITs were: SIT149 21.2% (33/151), SIT53 14.6% (22/151) and SIT26 9.6% (14/151). The most predominant lineage was T family 55.0% (83/151), Central Asia Strain 19.2% (29/151) and Haarlem consisted of 8.0% (12/151). Also, 5 (3.3%) isolates had no matching with the database.

Conclusion: The present study showed a high proportion of multidrug-resistant tuberculosis and pre-extensively drug-resistant tuberculosis among EPTB patients. The strain was mostly grouped into SIT149, SIT53, and SIT26. A large scale is required to define the molecular characteristics and drug resistance of *Mycobacterium tuberculosis* in extrapulmonary tuberculosis patients.

Keywords: Extrapulmonary Tuberculosis, Molecular typing, Mutations, multidrug-resistant tuberculosis and pre-extensively drug-resistant tuberculosis

1. Introduction

1.1. Background

Tuberculosis (TB) continues to be an important public health problem mainly in developing countries [1, 2]. Evidence indicated that one-third of the world's population has been infected by *Mycobacterium tuberculosis* (MTB) [3]. The world health organization (WHO) report is also indicated 10 million estimated new TB cases occurred worldwide in 2018; of which 10% of people were living with Human Immunodeficiency Virus (HIV) and about 74% reside in Africa [3]. Five countries: India, Indonesia, China, Philippines, and Pakistan are among the top ten countries in terms of death due to TB worldwide [2]. TB is found in every nation, but the majority of TB cases are concentrated in developing countries [3].

There are two types of TB based on the anatomical sites where the disease manifested. These are pulmonary TB (PTB) and Extra-pulmonary Tuberculosis (EPTB) [4, 5, 6, 7]. EPTB occurs in any part of the body except for the lungs. It becomes an important clinical problem because it accounts for about 15%–20% of TB burden [3]. The prevalence of EPTB is higher in patients co-infected with HIV, especially in endemic countries, though it is less infective than pulmonary tuberculosis [8, 9].

Various biological methods are employed to diagnose EPTB. These are smear microscopy, culture identification, histopathology, tuberculin skin test, serological assays, interferon-gamma release assays and nucleic, acid amplification (NAA) tests [10]. Smear microscopy is widely used in the diagnosis of EPTB but has drawbacks owing to low and variable sensitivity values and could not differentiate between MTB and non-tuberculous mycobacteria (NTM) [11, 12]. Culture identification for MTB also has variable sensitivities in different extrapulmonary specimens [13] with the turnaround time of 4 to 8 weeks and requires skillful technicians [14]. The diagnosis of EPTB from tissue samples is usually made by histopathological examination that depends on the presence of granulomatous inflammation and causes of necrosis. However, histology does not distinguish between EPTB and infections from other granulomatous diseases such as NTM, sarcoidosis, leprosy, and systemic lupus erythematosus [15].

The tuberculin skin test is also useful for the diagnosis of EPTB. However, false-positive reactions occur as a result of previous Bacille Calmette Guerin (BCG) vaccination or sensitization

to NTM, and false-negative results that occurred in the immune-compromised patients, elderly persons or overt forms of TB [12]. Moreover, in vitro T-cell-based interferon-gamma release assays have been used for the diagnosis of both latent and active TB, but these assays do not differentiate between latent and active TB infection [14].

Nucleic acid amplification tests (NAATs) require an expensive thermal cycler to amplify DNA fragments in multiple temperature-dependent steps. Therefore, some polymerase chain reaction (PCR) assays, such as Xpert MTB/RIF, are very costly, which is an obstacle to use in low-income countries. Loop-mediated isothermal amplification (LAMP) is also the molecular techniques used for TB diagnosis. It is an isothermal DNA method that relies on two or three sets of primers to amplify minute quantities of DNA within a shorter period of time. Compared with other NAATs, LAMP is cheap [15]. LAMP is a new assay with high accuracy for pulmonary TB detection [16]. However, in EPTB its accuracy varies from site to site of the organs where the disease manifested and LAMP has good diagnostic efficacy in EPTB detection [10]. In resource-limited countries, spoligotyping is remained an important tool to analyze the frequency of various *MTB* genotypes strains. It is a PCR based method for detection and typing of the MTB complex using a single chromosome locus with high polymorphism and the direct repeat (DR) region. It consists of a sequence of 36 pb which are separated by 43 non-repeated spacers. Spoligotyping offers great advantages such as identifying different strains which shows unique hybridization pattern and strains of an outbreak shared the same pattern [17]. The patterns obtained from direct examination of the clinical samples are identical to the pattern obtained from cultures of the same samples by DNA sequencing [18].

Genotype MTBDR*plus* and MTBDRsl are molecular-based assays designed to detect specific drug resistance encoding mutations in MTB have the advantage of achieving results within 48 hours, much faster than conventional drug susceptibility test (DST). The Hain line probe assays use reverse hybridization technology to detect mutations associated with resistance of tubercle bacilli to both first- and second-line anti-TB drugs. Genotype MTBDR*plus* allows for identification of Isoniazid (INH) and rifampicin (RIF) resistance by disclosing mutations in the katG, inhA, and rpoB genes, while MTBDRsl detects resistance to fluoroquinolones (FQs), aminoglycosides (kanamycin (KAN); amikacin (AMK); viomycin (VIO) and capreomycin (CAP)) by finding mutations in four different loci such as gyrA, gyrB, rrs and eis [19].

1.2. Statement of the problem

There are an estimated 10 million new cases of TB and an estimated 1.2 million people died in 2018 [2]. Multidrug-resistant TB (MDR-TB) remains a public health crisis and a health security threat across the world. World Health Organization (WHO) estimate indicated that 558 000 new cases of rifampicin resistance occurred in the globe and 82% of rifampicin-resistant cases develop MDR-TB. Moreover, about 9% of MDR-TB patients also have extensively drug-resistant TB (XDR-TB) [3]. The proportion of EPTB among all TB cases varies from country to country. Previous reports have shown that the proportions of EPTB in new TB cases are ranged from 17% to 52% across the world [20]. Moreover, the proportion of EPTB in both new and relapse TB cases in South Asia countries (Afghanistan, Pakistan, India, and Bangladesh) ranged from 19% to 23% [21].

Treating MDR-TB is more complex than treating drug-sensitive TB, as second-line TB drugs are more difficult to acquire, often require intravenous administration, and are more toxic and less effective than first-line TB drugs (2). Patients with MDR-TB also require longer progressions of more costly treatment and experience higher mortality than those infected with drug-sensitive TB (3). Moreover, it can take two years or more to treat MDR-TB, resulting in social isolation, loss of employment, and long-term socioeconomic and psychological effects

The primary drug-resistance in EPTB cases may be due to infection with primary drug-resistant strains spread out by pulmonary tuberculosis patients. Though EPTB does not spread from one patient to the other posing a threat of spread with primary drug-resistant strains, it causes serious complications unless diagnosed and treated earlier [8]. There are two ways by which people get drug-resistant TB. First, when TB treatment is inadequate, i.e., when patients fail to adhere to the treatment regimens and wrong drug prescription. Second, when there is a direct transmission of drug-resistant TB bacilli from one person to another and co-infection are increases drug-resistance [6]. Diagnosis of EPTB is difficult due to the paucibacillary nature of the disease (low bacterial load in the non-respiratory specimens), challenging sample collection, variable clinical presentation, and invasive procedures to obtain an appropriate sample and lack of laboratory facilities in resource-limited settings [9, 10].

TB is a major public health problem in Ethiopia. Ethiopia is one of the 30 high multidrug resistance TB (MDR-TB) countries and ranked 15th with more than 5,800 estimated new cases

each year [3]. A recent estimate indicated that the prevalence of MDR-TB is 0.71% in newly diagnosed patients and 16% in previously treated patients in Ethiopia [2]. In addition, a recent systematic review of local MDR-TB prevalence studies in Ethiopia included six eligible papers for analysis out of 23 studies done between1994-2012 and reported a high overall MDR-TB prevalence in the range of 3.3%-46.3%. Also, two studies done in Bahir Dar (northwest Ethiopia) in 2011 and Addis Ababa in 2005-2006 reported 1% and 4.4% XDR-TB respectively [6]. Besides, the prevalence of culture-confirmed extrapulmonary tuberculosis is 29.8% among extrapulmonary tuberculosis suspected cases in Ethiopia. The proportion of EPTB, with the majority of TB lymph nodes among newly diagnosed TB patients, has been increasing for the last two decades [22]. Despite the high burden of EPTB in Ethiopia, there is an information gap on drug resistance patterns and molecular characterization of EPTB. Thus, the current study was conducted to determine molecular characterization and drug resistance patterns of MTB complex among confirmed extrapulmonary tuberculosis cases in Addis Ababa, Ethiopia.

1.3. The rationale of the study

The information about molecular characteristics and DST of MTB among EPTB in Addis Ababa is limited. There is no strong evidence on the burden of drug resistance and molecular characteristics among EPTB patients in Ethiopia. The information is very crucial to provide an overview of molecular characteristics of MTB causing EPTB which is useful to understand the new strains that circulate in the population. So far only one study conducted in Addis Ababa using spoligotyping and Geno-Type MTBC to identify the strain and drug resistance [23]. However, in this study, LPA was performed to identify mutation type associated with drug-resistance for all culture-positive EPTB isolates, and further second-line anti-TB drug resistance was determined for RR/MDR EPTB isolates using SL-LPA, and phenotypic DST. Also, the above study has included participants from two hospitals only, but the current study was covered participants from eight hospitals which makes the current study more generalizable than the previous study (the sample size of the EPTB isolates three-fold of the previous one). The molecular characteristics among strains of MTB have a great effect on the pathogenicity and transmissibility, design for vaccines production, identification of genes for drug targets, and improving molecular diagnostic techniques. Therefore, this study was aimed to determine drug resistance patterns and molecular characteristics of MTB among confirmed extrapulmonary tuberculosis cases in Addis Ababa.

2. Literature review

A study reported from Brazil by Gomes *et al* on 612 isolates of EPTB cases indicated 506 (83%) SIT level, while 106 (17%) had orphan patterns, 297 (48%) belonged to the Latin-American Mediterranean family (LAM) family, 82 (13%) belonged to the T family and 45 (7%) belonged to the Haarlem family [24]. A cross-sectional study reported from India by Suzana *et al* is also indicated that 61 spoligo patterns, 128 clusters in the spoligotype database (spoldb4 database) with spoligo international type (SIT) number and 35 true unique isolates [25]. The most predominant spoligotype identified was EAI lineage (56) which followed by Beijing (28), CAS (20), T (9), U (7), X (3), H (3), BOVIS 1 BCG (1) and LAM (1) [25]. Out of the 163 isolates tested for resistance only 42 of the 163 MTB isolates had MDR and 25 Pre-XDR [25].

Similarly, another study reported from India by Kandhakumari *et al* revealed that 67 *Mycobacterium tuberculosis* isolated from a single EPTB specimen. Among 67 isolates with 41 spoligo patterns, 28 isolates (41.8%) were EAI lineage which was the predominated and followed by 18 orphans (26.9%), 10 Beijing (14.9%) and 8 U (11.9%) [26]. However, BOVIS1 BCG (ST482), T1-T2 (ST78), and H3 (ST50) strain each were represented by a single strain [26]. Still another study reported by Sankar *et al* from North India shown that 125 MTB strains isolate from patients with EPTB [17]. The spoligotyping results indicated that 110 (88%) displayed known patterns while 15 (12%) isolates had no matching database [17]. CAS family (57.27%) was the most predominant spoligotype that isolated. Moreover, 38 isolates were the largest clade and belonging to the CAS1 DEL lineage [17]. Thus CAS family lineage is the most prevalent genotype in the EPTB cases in the population [17].

The study reported from Italy indicated the association of MTBC isolates of BOVIS and CAS genotypic lineages with EPTB from 244 isolates [27]. Spoligotyping identified were ten major genotypic families, such as Africanum (1.4%), Beijing (7.2%), Bovis (1.7%), CAS (2.7%), EAI (4.5%), Haarlem (21.4%), LAM (12.8%), S (5.2%), T (28.2%), X (1.2%), and other spoligotypes undefined in SpolDB4 (13.8%) [27]. The distribution of EPTB among the genotype families was reported [27]. The study reported from Turkey also indicated that spoligotyping identified a total of nine spoligo superfamilies that include 344 (86.6%) of the 397 study isolates [28]. Fifty-three (13.4%) of the spoligotype was not matched to any spoligotypes in the SpolDB4 database [28]. Ill-defined T (46.3%, 184/397), LAM, (19.4%, 77/397), and Haarlem (H, 14.1%, 56/397) were

the most commonly seen super families reported in the same study [28]. Still another study reported from Turkey indicated that 132 different spoligopatterns were identified and 46 different clusters for 384 strains were determined [29]. The most predominant spoligotypes were ST53 (24.7%) and ST41 (8.1%) which followed by ST50 (5.7%), ST284 (4.7%), and ST4 (4.3%), respectively [29]. ST53 was the most predominant type in both sexes [29]. MDR was determined in 12 isolates, of which six were ST1 [29]. DST was applied for 396 patients and 14 strains showed resistance to RIF, with high-level resistance to INH in 8, low-level resistance to INH in 20, and MDR in 12, of which six were type ST1 [29]. The overall MDR proportion was 3% [29].

A study conducted in Mozambique has indicated 45 isolates were *Mycobacterium tuberculosis*, but none of *Mycobacterium bovis* was identified [30]. Cervical TBLN comprises 39 (86.7 %) of cases, which is the main cause of TBLN, and 66.7% of those cases were identified from people living with HIV [30]. Moreover, the result reported from Maputo a capital of Mozambique indicated that majorities of TBLN caused by a variety of *Mycobacterium tuberculosis* strains [30]. The most prevalent lineage identified was EAI 19 (43.2 %) [30]. The specific common spoligotypes identified were SIT 48, SIT 42 (LAM 9), SIT 1 (Beijing), and SIT53 (T1) [30].

The study reported by Wamala *et al* indicated clinicopathological features of TB due to *M. tuberculosis* Uganda genotype in patients with TB lymphadenitis [31]. Spoligotyping revealed that 54 distinct spoligotype patterns of which 33 (61% patterns were available in the SITVIT-WEB and 21(39%) patterns were not found in the database [31]. Moreover, Euro-American lineages were predominant (62%) which followed by CAS lineage (18%) [31]. There were also three Bejing strain and not *Mycobacterium bovis* [31].

A study reported from Ethiopia on molecular and drug sensitivity patterns of mycobacterial isolates from EPTB cases indicated that, out of 200 clinically suspected extrapulmonary tuberculosis patients, only one was *M. bovis* and 58 were MTB strains with 31 different spoligotype patterns grouped into seven clusters [23]. Euro American lineage (71.1 %), Indo Oceanic (5.1 %), East Asian (3.4 %) East Africa Indian (18.6 %) and *M. bovis* (1.6 %) [23]. Resistance to RIF was higher (22 %) than resistant to INH, streptomycin (STM) and Ethambutol (EMB) (8.1 %, 5 %, and 3 % respectively) [23]. Out of the 37 isolates tested for resistance, only 2 isolates were resistant for both STM and INH, but no MDR strain was found [23]. Moreover, a study reported from Dessie Hospitals, Northern Ethiopia identified 28 different strains [32]. Two

of these strains were SIT50 and SIT393 that consisted of three isolates each, but the remaining 26 strains were an orphan strain [32]. Classifying strains on the bases of phylogeny level (lineage) of MTB using SPOTCLUST software revealed that the strains belonged to Euro-American (EA) 57.1% (16/28), East-African Indian (EAI) 14.3% (4/28) and Indio Oceanic (IO) 28.6% (8/28) lineages [32]. A similar study reported from Debre Birhan Hospital from central Ethiopia revealed 16 clusters out of which 2 were new to the SITVIT database [33]. Of the 44 spoligotypes, 27 (61.4%) were from modern (Euro-American) and 16 (36.3%) were ancestral (Indo-Oceanic) tuberculosis lineages [33]. The most dominant spoligotypes were SIT54, SIT53, and SIT149 in decreasing order [33]. SIT54, SIT134, SIT173, SIT345, SIT357, SIT926, SIT91088, and SIT1580 were reported for the first time in Ethiopia [33]. The family with the highest frequency identified as MTB family was T1 [33]. Most of the strains belonged to EA and IO lineages [33].

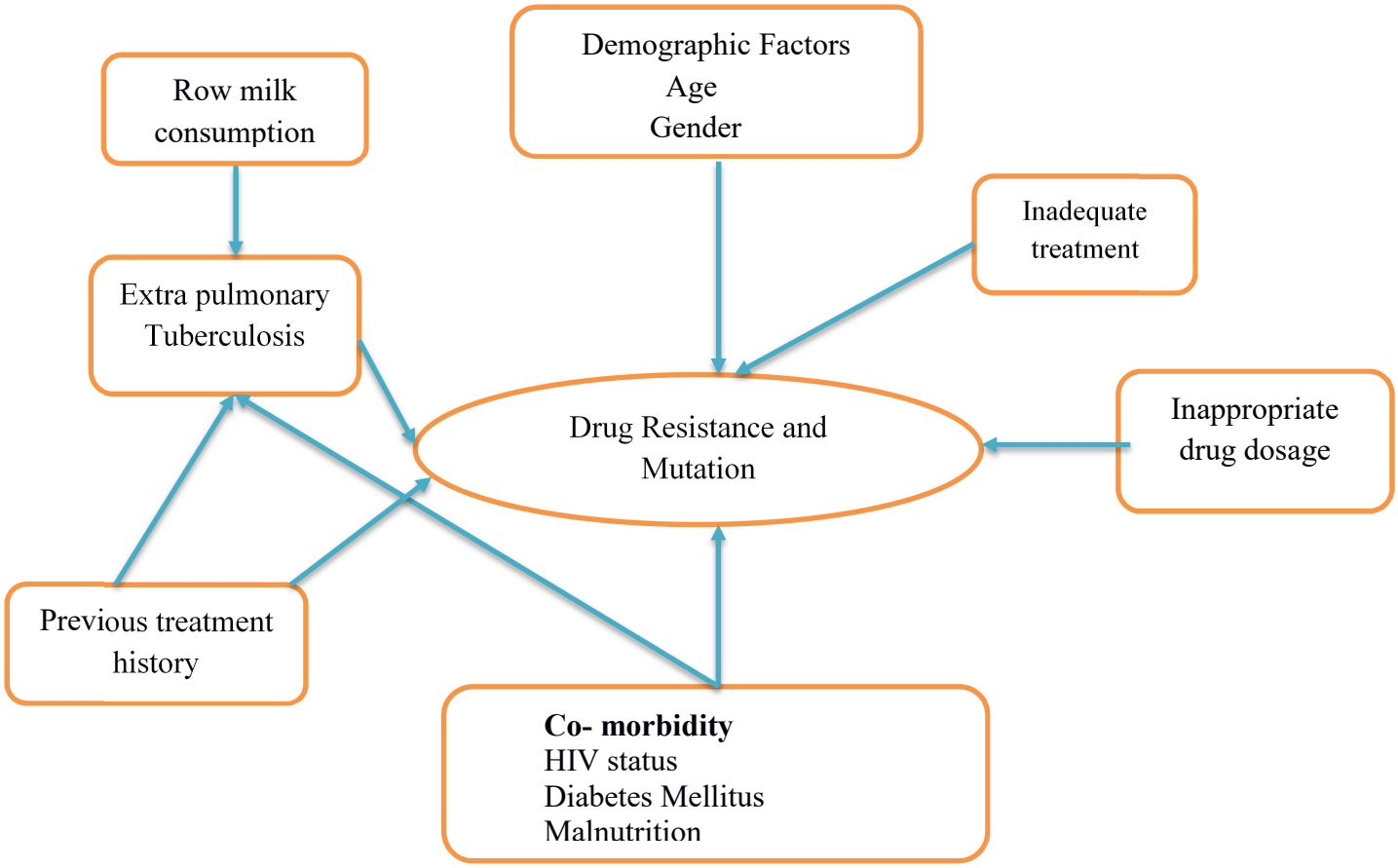


Figure 1: Conceptual framework of a risk factor for extrapulmonary tuberculosis and drug resistance [3, 8, 9.23, 24, 29, 32, 31, 33].

3. Objectives

3.1. General objective:

The main aim of this study was to determine molecular characterization and drug resistance patterns of *Mycobacterium tuberculosis* complex among confirmed extrapulmonary tuberculosis isolates in Addis Ababa, Ethiopia.

3.2. Specific objectives

- To determine the drug susceptibility profile of *Mycobacterium tuberculosis* complex in isolates obtained from extrapulmonary tuberculosis patients.
- To describe the molecular characterization of *Mycobacterium* species
- To determine the frequency of gene mutations associated with drug resistance of *Mycobacterium tuberculosis*.
- To compare the performance of LPA with MGIT 960 for the detection of drug resistance in extrapulmonary tuberculosis.

4. Hypothesis

Regarding variation in the molecular characterization profiles, there was no difference, but regarding with drug resistance patterns of *Mycobacterium tuberculosis* isolates of EPTB between the current study and a research done in Addis Ababa and Debrebirhan showing difference the burden of drug resistance in EPTB

5. Materials and Methods

5.1. Study area and study site

This study was conducted to analyze the molecular characteristics of MTBC among confirmed EPTB cases in Addis Ababa, Ethiopia. Addis Ababa is the capital city of Ethiopia which covers an area of 527 square kilometers. The city has estimated the total population of 3,384,569 according to the 2007 census [34]. Eight public hospitals such as Saint Paul Millennium Medical College hospital, ALERT Hospital, Armed Force Hospital, Black Lion Hospital, Ras Desta Damto Memorial Hospital, Zewiditu Memorial Hospital, Saint Peter Hospital, and Yekatit 12 Hospital were included in this study. Laboratory analysis was done at the National Tuberculosis Reference Laboratory (NTRL), Ethiopian Public Health Institute (EPHI). This laboratory operates under a biosafety level III based on the WHO recommendation for TB culture and DST tests. Among the tests at the NTRL TB culture, Phenotyping DST, Genotyping DST (Line Probe Assay and Xpert MTB/RIF Assay) tests, spoligotyping, and Geno-Type MTBC techniques are available for this research work.

5.2. Study design and Period

A laboratory-based cross-sectional study was conducted among confirmed EPTB cases in Addis Ababa, Ethiopia in a period between November 2019 and April 2020. Laboratory analysis was done at NTRL, EPHI. This study was the continuation of two studies; a study from the evaluation of Xpert MTB/RIF Assay for the diagnosis of EPTB [Unpublished data], and a study on the diagnostic accuracy of Xpert MTB/RIF assay and non-molecular methods for the diagnosis of tuberculosis lymphadenitis [35] conducted from January 2016 to August 2017.

5.3. The study population

Isolates were collected from patients diagnosed as EPTB cases in between January 2016 to August 2017 at eight referral hospitals in Addis Ababa, Ethiopia. The isolates were properly stored in a deep freezer (-80◦C) at NTRL of EPHI for further study.

5.4. Inclusion and Exclusion criteria

5.4.1. Inclusion criteria

- All culture-positive isolates presented with a correct patient identification number and had demographic data were included in this study.

5.4.2. Exclusion criteria

- NTM and contaminated EPTB isolates were excluded from this study.

5.5. Study Variables

5.5.1. Dependent variables

- MTB strains type, mutation type, and drug resistance profile

5.5.2. Independent variables

- Demographic variables such as age, sex, marital status, educational status, previous TB treatment history, diabetes mellitus status, and HIV serostatus were independent variables

5.6. Measurement and Data collection

5.6.1. Sample Size Determination

In this study, we have included all the available 151 isolates stored at EPHI laboratories

5.6.2. Sampling method

Special sampling technique was not used rather; all 151 available stored isolates that fulfilled the inclusion criteria were included in the study.

5.6.3. Sub-culturing of stored isolates collection

All culture-positive isolates were obtained from EPTB patients attended eight referral hospitals in Addis Ababa during the previous study pried were included in this study. These samples from which the culture-positive isolates obtained were collected as part of the routine diagnostic examination of suspected EPTB patients treated in the referral hospitals indicated above. Informed consent for the use of *MTB* isolates was obtained from each patient. Also, as part of the

routine clinical examination, information on the current and previous history of *MTB* infection, history of contact with TB case and history of receiving previous anti-tuberculosis treatment was extracted from laboratory request forms. Extra-pulmonary specimens were collected from 778 clinical presumptive EPTB patients for the evaluation of Xpert MTB/RIF Assay for the diagnosis of EPTB that was conducted in 2017 study and 152 clinical presumptive EPTB cases for Diagnostic accuracy of Xpert MTB/RIF assay and non-molecular methods for the diagnosis of tuberculosis lymphadenitis in 2016 study. All specimens were tested for MTBC by culture and Xpert MTB/RIF for both studies. Of 930 extrapulmonary samples, 151 (16.2%) culture-positive isolates were obtained. Thus, 151 stored isolates were used in this study. All stored isolates were sub-cultured in liquid culture media for the detection of *mycobacterium tuberculosis*.

5.6.4. Laboratory analysis

5.6.4.1. First-line phenotypic drug susceptibility test

Five first-line drugs such as streptomycin (STM), isoniazid (INH), rifampicin (RIF) ethambutol (EMB), and Pyrazinamide (PZA) were tested using Mycobacterium Growth Indicator Tube 960 (MGIT 960) system. DST was performed by Antibiotic Susceptibility Testing (AST) set with the proportional method recommended by the WHO (36). The concentrations of the drugs in media were: STM 1.0μg/mL, INH 0.1μg/mL, RFP 1.0μg/mL, EMB 5μg/mL and PZA 100μg/mL which consists a growth control tube for each drug contain tube. The bacterial inoculums were diluted to 1:100 before inoculation into the growth control tube and 0.5 mL bacterial suspension was added (37). The inoculated tubes were incubated in MGIT 960 system and monitored every one hour for an increase in fluorescence. For SIRE sensitivity MGIT 960 tubes were incubated for a maximum of 13 days and 21 days for PZA. [36].

5.6.4.2. Second-line phenotypic drug susceptibility test

Second-line DST was performed for all MDR-TB isolates using MGIT 960 systems. All liquid MGIT-positive MTB culture within 1 to 5 days were used for second-line DST. 800µl SIRE supplement and 100µl working drug solution were added into MGIT tube which contained 7ml modified Middlebrook 7H9. A working solution of each drug was prepared at the concentration level of: Ofloxacin (OFX) 2.0µg/ml, Capreomycin (CAP) 1.25µg/ml, Amikacin (AMK)

1.0µg/ml, Kanamycin (KAN) 2.5µg/ml, and Ethionamide (ETH) 2.5 µg/ml based on the manufacturer's recommendations [37, 38].

5.6.4.3. Geno-Type MTBDR*plus* and MTBDR*sl* assay

The MTBDR*plus* and the MTBDR*sl* assays were performed directly on the MTB isolates according to the manufacturer's instructions. The first DNA was extracted from *Mycobacterium tuberculosis* complex isolates. Next, PCR was performed using pre-made amplification mixes (amplification mix A and amplification mix B) that contained all the necessary components. Following amplification, DNA was hybridized with specific oligonucleotide probes immobilized on a strip which enables the detection of the presence of MTBC as well as simultaneously the presence of wild-type and mutation probes for resistance to RIF, INH, FQs and injectable drugs (CAP, AMK, VIO, and KAN). If a mutation is present in one of the target regions, the amplicon was not hybridized with the relevant probe. Mutations can be detected by a lack of binding to wild-type probes as well as by binding to specific mutation probes for the most commonly occurring mutations. Visualization was achieved using a streptavidin-alkaline phosphates conjugate-mediated colorimetric reaction leading to colored bands on the strip at the site of probe binding which can be read with the naked eye [19, 39, 40, 41].

5.6.4.4. GenoType MTBC

Differentiation of MTBC down to the species level was carried out by using genotype MTBC assay. Geno-Type MTBC is a molecular Genetic Assay for the differentiation of the MTBC from cultured materials. It is a qualitative in vitro test from cultured materials for the identification of species or strains belonging to the MTBC. The complexes are *M.tuberculosis/M.canettii, M.bovis, M.africanum, M.microti, Sub-species of bovis, M.caprae, M.bovis BCG.* The procedure involved in Genotype MTBC Hain molecular line probe assay includes the following method DNA Extraction, DNA Amplification, and Hybridization of the amplified DNA and these were done according to the manufacturer instructions [42].

5.6.4.5. Spoligotyping of MTBC

Spoligotyping of Mycobacterium tuberculosis Complex Isolates was carried out by use of Ligation-Based Amplification and Melting Curve Analysis. Spoligotyping was performed as described elsewhere [43]. Briefly, 5µl suspension of heat-killed mycobacterial cells was added

into 20µl a reaction mixture (a total volume of 25 µl) which contains 12.5 µl HotStar Taq Master Mix (Qiagen), 2 µl each primers (DRa and DRb), 3.5 µl molecular grade water and 5 µl suspension of heat-killed cells. The mixture was amplified DNA by PCR and heated for 10 minutes at 96^{o}C. The PCR products are labeled with biotin because of DRa primer was biotinylated. Then 25 µl of the amplified PCR product was mixed with 150µl primary buffer, loaded on the minibloter and hybridized for 60 minutes at 60^{o}C in the standard hybridizing oven to a set of 43 immobilized oligonucleotides, each cross ponding to one of the unique spacer DNA sequences within the directed repeat locus. After hybridization, the membrane was washed twice in 250 ml seconder buffer at 60^{o}C for 10 minutes on the shaking platform. Then, following by cooling the membrane in distilled water at roller bottle and add 7µl streptavidin peroxidase-conjugated for 60 minutes incubate at 42^{o}C in hybridizing oven. After this stapes, the membrane was washed twice in 250 ml by seconder buffer for 10 minutes at 42^{o}C by shaking which was followed by two times washing the membrane using 250 mL 2X SSPE (Saline-Sodium phosphate EDTA buffer) for 5 minutes at room temperature. Finally, Hybridized DNA was detected by the enhanced chemiluminescence detection system and by exposure to X-ray film per specification of the manufacturer and absence or presence of spacers was detected by visualizing the black spots developed on the membrane film [44, 45].

5.7. Data Quality Assurance

Quality was assured by using different approaches. Trained laboratory professionals were performed the molecular and drug susceptibility tests. Internal quality control was analyzed along with study clinical isolates. A known susceptible *MTB (H37Rv)* control and resistant isolate were tested by including them in each test run of drug susceptibility test for molecular characterization known susceptible *MTBC* (*H37Rv* and *BCG)* were run at each batch of the test as control. The sterility of the culture media was checked and a 10% confirmed result was repeated for all methods. Double data entries were done and data cleaning was made before the main analysis.

5.8. Data Analysis and Interpretation

The spoligotype patterns obtained were entered into the international spoligotyping database, and assign into the existing SIT (Spoligotype International Type) number. All data generated from the laboratory result were double entered using into SPSS version 23 statistical software. A

descriptive phenotypic, genotypic and epidemiologic data analysis was done. The level of statistical significance was set at $p \leq 0.05$.

5.9. Ethical consideration

Ethical clearance was obtained from the department of Research and Ethical Review Committee of Medical Laboratory Sciences, Addis Ababa University. The letter of collaboration with ethical clearance was submitted to EPHI to getting permission to use the required information and stored isolate. The study was conducted by stored clinical isolates anonymously, and confidentiality of the results was assured by keeping the documents in a locked area. The name of the client did not appear on the data collection template.

5.10. Dissemination of Results

After conducting the research, the results of the study were submitted to Addis Ababa University, College of Health Sciences and the Department of Laboratory Sciences. Also, the result of this study was presented as a technical report or orally to EPHI and other concerned bodies. The finding of the study was also presented to the medical scientific community and a manuscript was submitted to peer-reviewed journals for publication.

5.11. Operational Definitions:

Multidrug resistance (MDR): Resistant to at least isoniazid and rifampin, the two most potent TB drugs [2].

Pre-extensively drug-resistant TB (pre-XDR-TB): resistance to isoniazid and rifampicin and either a FQ or a second-line injectable agent but not both.

Extensively drug-resistant TB (XDR TB): resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs [19].

Extrapulmonary tuberculosis (EPTB): a case of TB that involves organs other than the lung e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones, and meninges [17].

6. Results

6.1. Demographic data of study participants

Of 151 isolates from EPTB patients included in this study, more than half of the study participants (54.3 %) were males. The mean age of the participants was 32.3 years (SD 17.32 years) with an age range of 4 to 78 years. The majority of participants were in the young-age adult groups 15 to 39 years. Most of the patients (90.7 %) had no previous TB treatment history, and 51.0% were attended primary school, 44.9% married and 30.5% were daily laborer. About 22.5% of the participants were HIV sero-reactive, and 9.3% had diabetes mellitus [Table 1].

Table 1: Demographic data of EPTB patients in (n=151), 2020

Characteristics		Frequency	Percentage (%)
Age in years	<15	16	10.6
	15-39	89	58.9
	40-59	33	21.9
	>60	13	8.6
Gender	Female	69	45.7
	Male	82	54.3
HIV status	Nonreactive	96	63.6
	Reactive	34	22.5
	Unknown	21	13.9
Marital status	Married	98	64.9
	Single	44	29.1
	Separated	9	6.0
Educational status	Illiterate	42	27.8
	Primary School	77	51.0
	Secondary School	24	15.9
	Higher Education	8	5.3
Occupation	House wife	13	8.6
	Daily laborer	46	30.5
	Government employee	12	7.9
	Unemployed	38	25.2
	Other	42	27.8
Diabetes Mellitus	Yes	14	9.3
	No	137	90.7
MDR-TB contact	Yes	7	4.6
	No	144	95.4
TB Category	New cases	137	90.7
	Re-treatment	14	9.3

*TB-Tuberculosis, *MDRTB-Multidrug Resistance Tuberculosis and *HIV-Human Immunodeficiency Virus

Figure 2 depicts the organ distribution of EPTB in 151 isolates included in this study. Based on the clinical presentation frequency distribution of different types of specimen for EPTB, 77.8 % of the enrolled patients fall into two major disease categories; 99 (65.6 %) tuberculous lymphadenitis or Lymph node aspirate, 32 (21.2 %) pleural tuberculosis

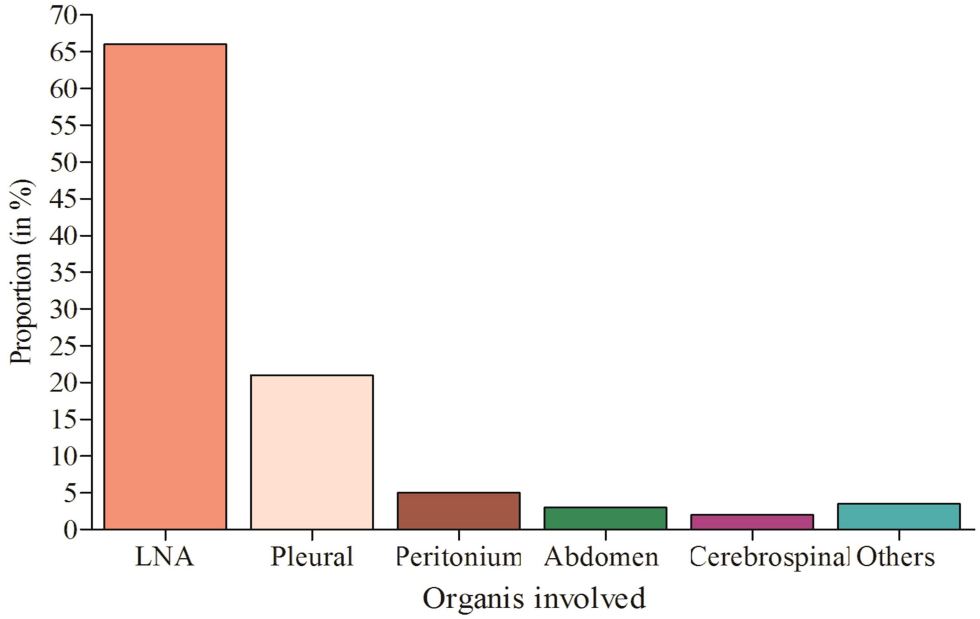


Figure 2: Frequency of different types of specimen for EPTB.

6.2. Phenotypic Drug sensitivity pattern of Mycobacteria for the first line

A total of 151 isolates from EPTB specimens were performed phenotypic drug susceptibility testing by BACTEC MGIT 960 for first-line drugs. Of 151 isolates, 85.4% (129/151) were sensitive to all first-line drugs: STM, INH, RIF EMB, and PZA. However, twenty-nine isolates were resistant to at least one or more drugs. Twenty-two (14.6%) of isolates were resistant to INH and similar numbers were resistant to PZA. Similarly, 14 (9.3%) of isolates were resistant to RIF, while nine (6.0%) resistant to STM and three (2.0%) were resistant to EMB. The overall proportion of MDR-TB was 9.3%. A higher proportion of MDR-TB was seen in previously treated TB cases than new cases [Table 2].

Table 2 Phenotyping drug resistance profiles for first-line drugs among confirmed EPTB isolates by Patient History profile, 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n %	Total n (%)
Total tested Isolate	137	14	151
Susceptible	118(86.1)	11(78.6)	129(85.4)
Resistance to any drug			
STM	7(5.1)	2(14.2)	9 (6.0)
INH	19(13.8)	3(21.4)	22 (14.6)
RIF	11(8.0)	3(21.4)	14 (9.3)
EMB	3(2.2)	-	3 (2.0)
PZA	19(13.9)	3(21.4)	22(14.6)
Mono resistance			
INH	2(1.5)	1(7.1)	3 (2.0)
PZA	8(5.8)	1(7.1)	9(5.9)
Resistance to more than one drug			
INH + RIF	11(8.0)	3(21.4)	14(9.3)
INH + PZA	12(8.6)	3(21.4)	15(9.9)
INH + STM	7(5.1)	2(14.3)	9(6.0)
INH + EMB	3(2.2)	-	3(1.32)
INH + RIF + STM	4(2.9)	1(7.1)	5(3.3)
INH + RIF + EMB	2(1.5)	-	2(1.3)
INH + RIF + PZA	8(5.8)	2(14.3)	10(6.6)
INH + RIF + EMB + PZA	1(0.7)	-	1(0.7)
STM + INH + RIF + PZA	4(2.9)	1(7.1)	5(3.3)
INH + RIF or MDR	11(8.0)	3(21.14)	14 (9.3)

*STM-Streptomycin *INH-Isoniazid * RIF-Rifampicin * EMB-Ethambutol * PZA-Pyrazinamide and *MDR-Multiple drug resistance

6.3. Second-line phenotypic drug sensitivity testing

Second-line drugs DST were conducted for a total of 14 MDR isolates. The second-line drugs for which DST conducted in this study include AMK, CAP, ETH, KAN, MOX, and OFX. Of 14 isolates 14.3% were resistant to MOX and 7.1% to CAP. Similarly, of 14 isolates 7.1% were resistant to ETH, while 14.3% resistant to OFX. Of 14 MDR-TB isolates, 21.4% were developed pre-XDR-TB. Of the three isolates that developed pre-XDR-TB, a high proportion (33.3%) was observed in previously treated cases while 18.2% (2/11) in new cases. Out of the 14 isolates, none of the cases had XDR-TB in the present study [Table 3].

Table 3: Second-line phenotypic drug sensitivity testing according to new cases and previously treated cases (n = 14), 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n (%)	Total n (%)
Total Isolate	**11**	**3**	**14**
Amikacin	-	-	-
Capromycin	1(9.1)	-	1 (7.1)
Ethionamide	1(9.1)	-	1(7.1)
Kanamycin	-	-	-
Ofloxacin	1(9.1)	1(33.3)	2 (14.3)
Moxifloxacin	1(9.1)	1(33.3)	2 (14.3)
Pre-XDR	2(18.2)	1(33.3)	3 (21.4)

*XDR-Extensively Drug Resistant

6.4. First Line Genotypic Drug Resistance Prediction

Of 151 isolates on which genotype MTBDR*plus* assay performed 130 (86.1%) were sensitive to INH and RIF. However, 20 (13.2%) isolates were resistant to INH, while 15 (9.9%) resistant to RIF. Out of 151 isolates, 14 (9.3%) were developed MDR-TB and 21 (14%) developed resistance to any drug by GenoType MTBDR*plus* assay [Table 4].

Table 4: The proportion of genotyping drug resistance from a total of 151 *Mycobacterium tuberculosis* complex isolates from EPTB patients, 2020

Characteristics	Frequency	Percentage
Susceptible	130	86.1%
INH resistance	20	13.2%
RIF resistance	15	9.9%
Any drug resistance TB	21	14.0%
MDR-TB	14	9.3%

*RIF-Rifampicin, *INH-Isoniazid, *MDR-Multiple drug resistance and *TB-Tuberculosis

Of the total of 151 isolates, 21 were found either RIF or INH resistant. An rpoB mutation indicates that 15 (9.9%) were resistant to RIF. The RIF resistant isolates were shown mutations at different amino acid positions. Of 151 isolates, 13 (8.6%) had an amino acid mutation at the S531L position, and one (0.7%) isolates had at H526Y position. Of a total of RIF resistant isolates, two isolates had mutations in codon 526-529 that indicated the absence of wild type band (WT7) and 13 isolates had mutations in codon 530–533 which show the absence of wild type band (WT8). Mutations in katG and inhA genes lead to INH resistance. Of a total of 151 isolates, 18 (11.9%) had amino acid mutation at the S315T1 position and one (0.7%) had at C15T position. Among the INH resistant isolates, a missed wild-type probe was observed only on one isolate at inhA WT1 [Table 5].

Table 5: Frequency of gene mutations associated with resistance to Rifampicin (rpoB) and Isoniazid (katG and inhA) in EPTB isolates by Genotype MTBDR*plus* assay (n=151), 2020

Gene	Band	Mutant probe	Number of strains (n)	Percentage (%)
rpoB				
	WT1	506-509	-	-
	WT2	510-513	-	-
	WT3	513-517	-	-
	WT4	516-519	-	-
	WT5	518-522	-	-
	WT6	521-525	-	-
	WT7	526-529	2	1.3%
	WT8	530-533	13	8.6%
	MUT1	D516V	-	-
	MUT2A	H526Y	1	0.7%
	MUT2B	H52D	-	-
	MUT3	S531L	13	8.6%
katG				
	WT	315	18	11.9%
	MUT1	S315T1	18	11.9%
	MUT2A	S315T2	-	-
inhA				
	WT1	-15	1	0.7%
	WT2	-8	-	-
	MUT1	C15T	1	0.7%
	MUT2	A16G	-	-
	MUT3A	T8C	-	-
	MUT3B	T8A	-	-

* MUT-mutant and *WT-wild type.

6.5. Second Line Genotypic Drug Resistance Prediction

Fifteen (9.9%) RR-TB strains were identified among the 151 *M. tuberculosis* isolates used for first-line DST. To determine the number and percentage of pre-XDR and XDR strains, we performed DST for second-line drugs on all available MDR strains in this study. Of the total 15 MDR-TB or RR-TB isolates, two isolates were resistant to fluoroquinolone drugs and only one isolate was resistant to the injectable drugs alone. Of 15 isolates three (20%) isolate was identified as pre-XDR strains and XDR strain was not found from MDR-TB [Table 6].

Table 6: The proportion of second-line drug resistance by genotyping from EPTB patients (n=15), 2020

Drug resistance pattern	Isolates from new cases, n (%)	Isolates from previously treated cases, n (%)	Total n (%)
Total Isolate	**12**	**3**	**15**
Injectable drug	1(8.3)	-	1(6.6)
Fluoroquinolone drug	1(8.3)	1(33.3)	2 (13.3)
Pre-XDR	2(16.6)	1(33.3)	3 (20)
XDR	-	-	-

*XDR-Extensively Drug Resistant

The analysis of gyrA gene on 15 RR-TB isolates was revealed that one (6.6%) isolates had a mutation at codon A90V, while another one (6.6%) had a mutation at codon D94G, (6.6%) had a mutation at codon D94H, while another one (6.6%) had variable mutations at codon S91P. In gyrA gene analysis the most frequent mutations were the absence of bands WT3 in three (20%) and WT2 in two (13.3%) isolates. Other mutations were assessed in rrs gene for second-line injectable drug resistance. The rrs gene analysis was revealed that one (6.6%) isolates had a mutation at codon a1401g and the absence of bands WT1 in one (6.6%) isolates [Table 7].

Table 7: Frequency of gene mutations associated with resistance fluoroquinolone resistance (gyrA) and injectable drug resistance (rrs) by Genotype MTBDRsl assay (n=15), 2020

Gene	Band	Gene region or mutation	Number of strains (n= 15)	Percentage (%)
gyrA				
	WT1	85–89	-	-
	WT2	89–93	2	13.3%
	WT3	92–96	3	20%
	MUT1	A90V	1	6.6%
	MUT2	S91P	1	6.6%
	MUT3A	D94A	-	-
	MUT3B	D94N or D94Y	-	-
	MUT3C	D94G	1	6.6%
	MUT3D	D94H	1	6.6%
gyrB				
	WT1		-	-
	MUT1	N538D	-	-
	MUT2	E540V	-	-
Rrs			-	-
	WT1	1400	1	6.6%
	WT2	1484	-	-
	MUT1	A1401G	1	6.6%
	MUT2	G1484T	-	-
Eis				
	WT1	-37	-	-
	WT2	-10 to -14	-	-
	WT3		-	-
	MUT1	C-14T	-	-

Note: Data are presented as *MUT = mutant; *WT = wild type.

6.6. Comparison of genotypic DST result with phenotypic DST result of *M.Tuberculosis*

Based on the analysis of drug susceptibility results by genotypic and phenotypic DST methods, of 151 total isolates three (2.0%) *MTB* isolates had discordant results between the genotypic MTBDR*plus* test and the phenotypic DST. The common pattern of disagreement was susceptible in the genotypic DST analysis, while resistant in the phenotypic DST for INH (n=2). Also, one isolate was resistant in the genotyping DST analysis, whereas susceptible to phenotyping DST for RIF (n=1). The concordances of the GenoType MTBDR*plus* assay and the MGIT DST for the detection of INH and RIF resistance were 90.9% (20/22) and 100% (14/14), respectively. Kappa agreement between INH genotypic DST and phenotypic DST was 0.95. Besides, the kappa agreement between RIF genotypic DST and phenotypic DST was 0.96. Genotypic DST was compared with phenotypic DST as a gold standard, Line probe assays showed high the sensitivity, specificity, PPV, and NPV of LPA for INH were 90.9%, 100%, 100%, 98.5%) respectively. The respective values for RIF were 100%, 99.3%, 93.3%, and 100% (Table 8).

Table 8: Comparison of genotypic with phenotypic DST of *M. tuberculosis*, 2020

GenoType MTBDRplus assay		Phenotypic MGIT DST result					
		Susceptible	Resistance	Sensitivity	Specificity	PPV	NPV
INH	Susceptible	129 (85.4%)	2 (1.3%)	90.9%	100%	100%	98.5%
	Resistance	-	20 (13.2%)				
RIF	Susceptible	136 (90.1%)	-	100%	99.3%	93.3%	100%
	Resistance	1 (0.4%)	14 (9.3%)				

*RIF-Rifampicin, *INH-Isoniazid *DST-Drug Susceptibility Test, *PPV-Positive predictive value, *NPV-Negative predictive value, *MGIT-Mycobacterium Growth Indicator Tube, *MTBDR- Mycobacterium Tuberculosis Drug Resistance

6.7. Differentiation of MTBC (GenoType MTBC) species

Out of 151 MTBC isolates, 148 (98.0%) were identified as *M. tuberculosis* while three (2.0%) *M.bovis*. Other members of MTBC such as *M. africanum* and *M. microti* were not identified [Table 9].

Table 9: Frequency of MTBC species differentiated by GenoType MTBC Assay (n= 151), 2020

MTBC species	Number	Percentage (%)
M. tuberculosis	148	98
M. africanum	-	-
M. bovis	3	2
M. microti	-	-

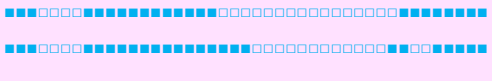
*MTBC-*Mycobacterium tuberculosis complex, *M. tuberculosis-Mycobacterium tuberculosis, *M. africanum -Mycobacterium africanum, *M. bovis-Mycobacterium bovis, *M. microti-Mycobacterium microti*

6.8. Strain typing result (spoligotyping)

All 151 MTB isolates were characterized by spoligotyping. Among these 151 MTB isolates characterized by spoligotyping, 146 (96.7%) displayed known patterns while 5 (3.3%) isolates had no matching with the international spoligotype database (SITVIT2). Of these isolates, 41 different spoligotype patterns were identified. The 146 (96.6%) isolates grouped into 11 families. The most predominant strain types were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%). The most predominant lineages were T family (55.0%), Central Asia Strain (19.2%), Haarlem (8.0%), and Orphan (2.7%). The remaining five (3.3%) isolates were not found in the database SITVIT2 and did not show similarity to any other known lineages and were considered as possibly new genotypes [Table 11].

Table 10: Spoligotyping pattern, octal codes, SIT and lineage of extra pulmonary *Mycobacterium tuberculosis* complex isolates (n = 151), 2020

Lineage /Family	SIT	No. (%)	Octal Number	Spoligotype pattern
Beijing	1	1(0.7)	000000000003771	
X1	336	1(0.7)	777776777760731	
Turkey	41	1(0.7)	777777404760771	
Haarlem		**12(8.0)**		
H1	47	1(0.7)	777777774020771	
H3	116	1(0.7)	777767775720771	
H3	121	1(0.7)	777777775720771	
H3	3	2(1.3)	000000007720771	
H3	3134	1(0.7)	777737377720771	
H3	50	2(1.3)	777777777720771	
H3	764	4(2.6)	777757777720771	
Bovis		3(2.0)		
Bovis1	694	2(1.3)	777776777760731	
Bovis1	820	1(0.7)	676763777777600	
T		**83(55.0)**		
T1	1129	2(1.3)	776777777760771	
T1	131	9(6.0)	777717777760771	
T1	393	3(2.0)	777757777760771	
T1	3315	1(0.7)	376777737760771	
T1	53	22(14.6)	777777777760771	
T2	52	2(2.3)	777777777760731	
T2	875	2(1.3)	777717777760731	
T2	Orphan	2(1.3)	017777777760731	
T3	1745	1(0.7)	773737777760771	
T3	37	6(4.0)	777737777760771	
T3-ETH	149	33(21.2)	777000377760771	
Ethiopia		**5(3.0)**		
Ethiopia	1729	1(0.7)	700000004177771	
Ethiopia	910	4(2.6)	700000007177771	
Ural		**6(4.0)**		
Ural-1	777	4(2.6)	777777777420771	
Ural-1	Orphan	2(1.3)	777777747420771	
Not defined	New	1(0.7)	777777770000771	
Not defined	New	1(0.7)	700000017774771	
Not defined	New	1(0.7)	777774777420771	
Not defined	New	2(1.3)	770000037760771	
Latin American Mediterranean		**5(3.3)**		
LAM9	1176	2(1.3)	777757607760771	
LAM9	42	3(2.0)	777777607760771	
East African Indian		**1(0.7)**		

EAI5	126	1(0.7)	477777777413771	
Central Asia Strain		29(19.2)		
CAS1-Delhi	22	7(4.6)	703777400001771	
CAS1-Delhi	25	2(1.3)	703777740003171	
CAS1-Delhi	26	14(9.3)	703777740003771	
CAS1-Delhi	289	3(2.0)	703777740003571	
CAS1-Delhi	357	1(0.7)	703777740000771	
CAS1-Delhi	429	1(0.7)	703777740003731	
CAS1-Delhi	794	1(0.7)	703757740003771	

7. Discussion

This study aimed to determine the molecular characterization and drug resistance patterns among EPTB in Addis Ababa, Of 151 isolates, twenty-two (14.6%) of isolates were resistant to INH and similar numbers were resistant to PZA. The overall proportion of MDR-TB was 9.3%. Of 14 MDR-TB isolates, 21.4% were developed pre-XDR-TB. A rpoB mutation indicates that 15 (9.9%) were resistant to RIF. Eighteen (11.9%) had amino acid mutations at the S315T1 position. Three (2.0%) *M. tuberculosis* isolates had discordant results between the genotypic MTBDR*plus* test and the phenotypic DST. We have identified three (2.0%) *M.bovis.* The most predominant lineages were also T (55.0%), Central Asia Strain (19.2%), and Haarlem (8.0%). The most predominant strain types were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%).

We have also investigated demographic, clinical and microbial characteristics of 151 EPTB isolates. In this study, the proportion of lymph node TB was the most common site (65.6%) which followed by pleural TB (21.1%). Also, most of EPTB were associated with specific young-age adult groups from 15 to 39 years. This observation is relatively similar to the findings of previous studies in Ethiopia [23] and India [25]. In our study, male sex was slightly more affected by EPTB than females (54.3% vs. 45.7%). This observation is consistent with the findings of previous studies reported in northern Ethiopia [32], Mozambique [30], and Turkey [28]. This might be due to under-diagnosis of TB in females as a result of various social and/or cultural factors, including their consequent impaired access to health care.

In this study, we were performed liquid DST for STM, INH, RIF, EMB and PZA. Also, second-line drug sensitivity testing was performed for AMK, CAP, ETH, KAN, MOX, and OFX for all MDR-TB isolates. In this study resistance against INH (14.6%) was the most frequent. This proportion is to some extent higher than an earlier report in Ethiopia [23] and Thailand [46]. However, our finding is similar to the results reported from India (13.3%) [47], and South Korea (12.8%) [49]. Moreover, two studies reported from India indicated higher (more than 30%) resistance against INH [48, 50] than our finding in which only 14.6% were resistant to INH. This difference could be due to the study area, sample size. Unlike our study conducted on a statistical optimum sample size the two studies reported from India were conducted on small sample size.

The most frequent resistance proportion was observed against PZA (14.6%). However, since there is no sufficient data on PZA resistance of EPTB globally, we could not compare it with the previous study. This study could serve as a reference for further study on resistance against PZA in EPTB patients. Although the proportion of PZA resistance is high in *MTB* cases, the phenotypic PZA susceptibility test is rarely performed due to technical difficulties [51]. PZA is active only at low pH which requires acidic culture medium for susceptibility testing. The acidic nature of the culture medium prevents about 20 to 25% of isolates from growing [51]. On the other hand, a large inoculum size also can cause alkalization of the medium that leads to false PZA resistance [51]. Due to this technical challenge in the laboratory and the possibility of false resistance most TB laboratories do not perform PZA susceptibility testing.

In our study, 9.3% of isolates were tested resistant against RIF which is in agreement with the previous study reported India in which 10.1% resistant against RIF [47]. However, higher resistance against RIF was reported from Ethiopia and India [23, 48, 50]. Relatively lower RIF resistance proportion was reported from India and Thailand [47, 46]. This variation in resistance against RIF might be due to geography and the use of different sample sizes.

In our study, 9.3 % of isolates were diagnosed with MDR-TB. Among all MDR-TB cases, 21.14 % were from previously treated patients and 8.0 % were from newly diagnosed cases. Previous studies reported from India indicated relatively higher MDR-TB cases [48, 47, 50] than our findings. In contrast, two studies from South Korea [49, 52] and one study from Thailand [47] are reported lower MDR-TB proportion than our findings. The possible reason for high-level drug resistance in our findings than the previous studies results is due to non-adherence, loss to follow up, poor drug supply chain management, and poor quality of drugs [56]. Moreover, high MDR-TB in our study might be due to that the study was conducted among the population of TB or MDR-TB suspected patients included in the studies. However, our finding is relatively consistent with the findings of previous studies reported from India, China, and South Korea [50, 57, 54, 55, 49].

The result of high MDR-TB is one of the growing problems which have led to an increase in other drug-resistant cases of TB such as pre-XDR-TB and XDR-TB cases. For this reason, MDR-TB cases should be timely monitored. The proportion of pre-XDR-TB cases among MDR-TB patients in this study was 20%. A similar study was low reported Fluoroquinolones resistance in

Pakistan the rate of 6.0% [53]. The findings of this study are relatively similar to studies reported from India in which the proportions of pre-XDR-TB among MDR-TB patients were 18.4% [57]. On the other hand, the study reported from India indicated a higher proportion (38.2%) of pre-XDR-TB among MDR-TB patients [58]. In our study, the higher (20%) pre-XDR-TB was resistance against FLQ than injectable second-line drug-resistant. A study reported from India indicated a higher proportion (38.2%) of resistance against FLQ [58] than our finding of pre-XDR-TB. In contrast, another study reported from India indicated a lower proportion (3.4 %) of pre-XDR-TB that resistance to FLQ [47] than our study finding. The reason for the higher number of pre-XDR-TB cases in MDR-TB found in this study might be due to treatment failure that intensified the population drug-resistant strains [58]. Moreover, the possible explanation might be antibiotics are previously used for treating pneumonia which increases the resistance against these drugs. For example, fluoroquinolones and other drugs are broad-spectrum antimicrobial agents that widely and indiscriminate used particularly at sub-therapeutic doses. The wide use of these drugs most likely leads to an increase in quinolone-resistant. Thus, the indiscriminate use of these antibiotics might be contributed to the high proportion of pre-XDR-TB cases in this study [60].

Understanding the nature and frequency of mutations associated with drug resistance in *MTB* is important for the development and genetics-based assays for the diagnosis of drug resistance [61]. Early detection of drug resistance mutations would assist TB patient management and avoid treating individuals with inefficacious regimens [62]. The MTBDR*plus* and MTBDR*sl* assay have been widely used in clinical routines for the identification of MTB complex and detection of first and second-line drug resistance due to its shorter turnaround time. The direct use of the assay on clinical specimens is another key advantage, as this prevents waiting for cultures to grow.

Rifampicin resistance is related to mutations in a restricted area of the rpoB gene. In the Genotype MTDR*plus* test, Rifampicin resistance is related to the absence of one or more bands WT (WT1 to WT8) or the presence of the mutation band (MUT1, MUT2A, MUT2B, and MUT3) in area 500-531 of the rpoB gene [63]. In the present study, the most frequently observed mutations on the gene rpoB were the absence of bands WT8 (8.6%) and WT7 (1.3%). The presence of mutation on S531L and H526Y was lower than the previous studies reported from Iran and India [64 65 66]. However, our study finding was similar to the previous study reported from Sudan on S531L and H526Y mutations [67]. All RIF resistant isolates in this study were reported to have their

mutations rifampicin resistance determining region of rpoB codons 516 to 533. This finding is in agreement with a study reported in Iran [64].

The higher frequency of resistance to INH occurred due to mutation of the katG gene, whereas the lower frequency of resistance was caused by the mutations in the promoter region of the inhA gene [68]. In our study, from all INH resistant strains, 11.9% of strains had S315T1 mutation in katG region that led amino acid serine substitution to threonine. This was smaller than the result found in Northern India where 94.5% of isoniazid resistance isolates had a mutation in the katG gene [65]. Still studies reported from India also indicated mutation in codons S315T1 of katG 100% association with INH resistance [61, 66]. Moreover, the study reported from Sudan indicated that S315T1 mutation and mutation in the inhA gene that occurred in C15T are associated with INH resistance [67]. However, the same study reported in India is shown a higher proportion of C15T mutation [65]. Similarly, a study reported from Sudan found C15T mutation that resulted in INH resistance [67]. These findings are in agreement with our results in which mutation of C15T associated with INH resistance

As the burden of XDR-TB is increasing in MDR-TB patients, there is an urgent need for rapid and accurate tests for XDR-TB. XDR-TB is caused by strains of *MTB* that are resistant to INH, RIF, and any of the FLQs and at least one second-line injectable agent [69]. In our study, we found 20% of FLQ resistance among the 15 MDR-TB or RR-TB strains. Our finding is similar to the studies reported from Bangladesh [70] and India [57] in which FLQ resistance proportions were 19.11% and 18.4% respectively. The finding of our study is also corresponding with the global FLQ resistance proportion which is 17% among MDR-TB strains in 2014 [71]. Also, another study reported from New Delhi, India indicated a similar proportion (17.1%) of FLQ resistance among MDR-TB patients with our results (20%). Moreover, hospital-based studies reported from Wuhan, China found 22% of OFX resistance in TB suspects [72, 73]. In our study mutation in codon at gyrA was detected in 20% of FLQ resistant isolates. This mutation confers resistance to levofloxacin and is associated with low-level resistance to moxifloxacin [74]. In the present study, gyrA mutations were predominantly found to occur in codons 89–93, 92–96, S91P, and A90V. These most common mutations are largely corroborating the findings of the previous study [75]. The predominant rrs gene mutation was A1401G (6.6%). This is a common mutation reported in several studies to be associated with high-level resistance to AMK, KAN, and CAP

[76, 77]. In our study the absence of A1401G mutation was found in the gyrB and eis gene among MDR strains. The mutations in the gyrB gene are usually associated with low-level resistance to fluoroquinolones and are not as common as those in the gyrA gene [78]. This could be due to the limited number of resistant isolates to second-line drugs.

In the current study, we compared the performance of the line probe assay with the MGIT 960 system for the detection of drug susceptibility tests. In our study, DST results by BACTEC MGIT 960 were used as the 'gold standard' as this method has detected resistance of M. tuberculosis isolates to all drugs accurately. On two (1.3%) isolates discordance between molecular and phenotypic methods in drug susceptibility was confirmed for INH. Of these isolates, phenotypic DST has revealed resistance to INH, while genotypic provided susceptible results. Discordant results between phenotypic DSTs and genotypic DST may not be due to all mutations conferring resistance to anti-TB drugs being included in LPA assay. Resistant mutations may result in variable have low, moderate, or high phenotypic expression of drug resistance to INH. Silent mutations do occur at the genetic level with no change in drug susceptibility patterns. The LPA assay may miss silent or neutral mutations that result in phenotypical susceptibility. This could have been due to unidentified mutation in some other genomic region (like ahpC, kasA, furA) which is not targeted by this assay [79].

One (0.7%) isolate was shown resistance to RIF in the genotyping DST, but susceptible in phenotyping DST. The discordant results of DSTs for RIF between the genotypic MTBDR*plus* test and the phenotypic test result is our finding is similar to findings reported from Bangladesh and Congo Kinshasa [80, 81]. This could be due to the presence of mutations outside of the rpoB hotspot region to the target region for MTBDR*plus* assay. Another resistance mechanism such as hetero-resistance through mixed patterns of WT and mutant melt probe might be present. This could be because of some rare mutations occurring outside the 81bp region of the rpoB gene which is not targeted in our assay [79].

In this study, we compared the sensitivity and specificity of the line probe assay with the MGIT 960 system for the detection of drug susceptibility to first-line drugs. Line probe assays showed high sensitivity and specificity of the detection of susceptibility to RIF (sensitivity 100% and specificity 99.3%) and INH (sensitivity 90.9% and specificity 100%). In this study, relatively low sensitivity and specificity were observed in India for the detection of RIF (sensitivity 92.7% and

specificity 99.3%) and (sensitivity 95.2 and specificity 100%) [82, 83]. The specificity of 99.3% in the case of rifampicin resistance is in agreement with the previous reported was 97.30% [66].

Sensitivity and specificity of 99.3% and 100% for the detection of INH resistance are lower with the studies in India where a sensitivity of 93% and specificity of 97% were seen [77]. The sensitivity of the GenoType MTBDR*plus* assay for the detection of INH resistance in the present study was lower than those reported by previous studies in Ethiopia [84, 85] Uganda [86] and Pakistan [87]. The MTBDR*plus* assay was not able to detect the INH resistance in the two isolates, which were detected by the MGIT 960 system. This could be due to mutations in the inhA promoter and the ahpC-oxyR intergenic regions, which have not been included in the strips [88] or it could also be due to unidentified mutation. The high specificity of the MTBDR*plus* assay in detecting INH resistance and RIF resistance isolates agrees with the specificity of previous studies [84-86]. RIF resistance in one isolate was detected by the MTBDR*plus* assay but not by MGIT 960 system. Being susceptible by phenotypic method but resistant by the genotypic method may be associated with false RIF resistance due to a silent mutation which makes the probe fails to hybridize on a strip and interpreted as RIF resistant [89]. Recent studies have shown that these uncommon gene mutations may be susceptible or have low-/high-level resistance to isoniazid. We suggest the WHO recommends that DSTs of both phenotypes and genotypes should be used for all DST of first-line anti-TB drugs for diagnosis [90, 91].

In this study, molecular characterization of the strains of *M.tuberculosis* using spoligotyping identified 41 different spoligotype patterns from 151 isolates. Out of 151 isolates, 146(96.7%) patterns have matched a preexisting shared type in the SITVIT2 database, whereas the remaining 5(3.3%) patterns were not found in the SITVIT2 database. Our results of 151 MTBC isolates was shown a high diversity of strains such as Beijing, X1, Turkey, Haarlem, Bovis, T, Ethiopia, Ural, Central Asia Strain, East African Indian, and Latin American Mediterranean. There were also some orphan strains assigned to their most appropriate lineage and sub-lineage using the TB insight database. Most of the lineage highly prevailed in the current study were T (55.0%), CAS (19.2%), and H (8.0%). The most predominant strain spoligotypes were SIT149 (21.2%), SIT53 (14.6%), and SIT26 (9.6%). The occurrence and distribution of these families vary from region to region in Ethiopia. These results are compatible with two earlier studies reported from Ethiopia [27, 33]. The study reported from central Ethiopia indicated a high proportion of SIT53 and

SIT149 strains [33, 92]. Moreover, a study reported from Addis Ababa shown a high proportion of SIT53 strain and CAS family strains [27]. The number of SIT149 isolates in our study was thrifty two isolates higher the number registered in the SITVIT2 international database. This might be due to the association of this familiar strain with East African countries, especially in Ethiopia. Also, the presence of genetic diversity in the present study could be since the strains may have entered by the traders and other activates from the different regions as the study included participants from a town nearby Addis Ababa.

In our study, 2.0% of *M.bovis* was identified by GenoType MTBC Assay and Spoligotype pattern. The previous study has detected *M.bovis* in extrapulmonary TB [30] which is similar to our finding. Moreover, similar findings were reported previously from Ethiopia in which the proportion of *M. bovis* is 1.7% [23]. Besides, the study reported from India on extrapulmonary tuberculosis patients indicated 1.5% of the isolates had *M. bovis* [26]. The low proportion of *M.bovis* in this study could be attributed to the fact that participant's occupational background in this study was not associated with livestock or farms. This is because *M. bovis* strain is spread to a human through contact between to infected domestic animals such as cattle, wild animals, and ingestion of unpasteurized milk or contaminated meat. Also, the second usual route of infection with *M. bovis* is through animals and humans inhaling infected droplets that are expelled from the lungs by coughing.

Our study identified five isolates are new spoligotype patterns of *Mycobacterium tuberculosis* which were not present in the international database. This indicates that there is little information about *MTB* strains circulating in the study area. Since only a small number of isolates were characterized in our study, it is not possible to appreciate the diversity of *MTB* circulating in the study area. More isolates and wider geographical coverage are required to have a detailed insight about the *MTB* strains circulating in Addis Ababa.

8. Strength and limitation of the study

8.1. Strength

The strengths of this study were including different types of clinical specimens for EPTB patients and among the few studies that reported drug resistance and molecular characterization of EPTB in Ethiopia.

8.2. Limitation

The main limitation of this study was the lack of detailed patients' characteristics to identify risk factors associated with drug resistance in patients infected with EPTB. Also, the being participants were from a single city limited to the generalizability of this study finding at the national level. However, despite the limitations stated above, we do believe that the results of this study were less likely to be influenced by those limitations.

9. Conclusion and Recommendation

9.1. Conclusion

The present study provides an overview of molecular characterization and drug resistance *MTB* from extrapulmonary patients in Addis Ababa. In this study about three-fourth of the isolates shown T and Central Asia strain lineages. A high proportion of INH and PZA resistance, MDR and Pre-XDR were identified in EPTB isolates. The most mutations associated with first-line drug resistance occurred at catG gene (codon S315T1) region, while second-line drug resistance occurred at gyrA gene (codons 92–96).

9.2. Recommendations

The number of isolates obtained for this study was small and sample collection was limited to one city, Addis Ababa, which is limited to the generalizability of our results to the national level. Thus, large scale future study on molecular characterization and drug resistance of *MTB* in EPTB is required. Furthermore, the use of more accurate molecular techniques that could discriminate the mutation regions is recommended. High drug resistance proportion detected in this study also demands against immediate action to prevent the further occurrence of drug resistance. Resistance to PZA and INH was high indicating further investigations is required to know the exact cause and the possible contribution of isoniazid preventive therapy.

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10. Annexes

Annex 1 Protocol for Phenotypic DST

Purpose

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

Principle

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

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

Procedure BACTEC MGIT 960 culture

1. Label BACTEC MGIT 960 tubes with isolate number.

2. Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA
3. Add up to 0.5 ml of a well-mixed isolate to the appropriately labeled MGIT tube.
4. Incubate MGIT tube entered in the BACTEC MGIT 960 instrument after scanning each tube
5. Select Positive isolates for SD Bioline TB Ag MPT64 Rapid test SD Bioline TB Ag MPT64 Rapid test
6. Label each cartridge with the sample identification number
7. Place 100µL of the prepared bacterial culture on the specimen placing area of the test cartridge. Pipette tips should be changed between samples
8. A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Line
9. The formation of a purple to red line on the reading areas labeled [T] and [C] of the cartridge indicates a POSITIVE result
10. The right section of the result window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Line
11. The formation of a purple to red line on the reading area labeled [C] of the cartridge but not [T] indicates a NEGATIVE result
12. If no line is observed on the reading area [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new cartridge 13. Examine the reading area of the test plate after 15 minutes

Drug Susceptibility test using BACTEC MIGT 960

1. Select a positive isolates and ready for DST (For the preparation of the test inoculum, a positive 7 mL MGIT tube should be used the day after it first becomes positive on the BACTEC MGIT 960 instrument (Day 1), up to and including the fifth day (Day 5) after instrument positivity.
2. The tube is a Day 1 or Day 2 positive, mix well and proceed to “Inoculation Procedure for Susceptibility Test.” If the tube is a Day 3, Day 4, or Day 5 positive, mix well then dilute 1 mL of positive broth in 4 mL of sterile saline (1:5 dilution). Use the diluted suspension for the inoculation procedures.
3. Label each MGIT tube with relevant drug and laboratory number and date

4. Reconstituting lyophilized antibiotics in distilled water and MGIT tubes supplemented with 0.8 ml of the enrichment solution (BactecMGIT SIRE supplement; Becton Dickinson), the critical concentration for each drug was 5.0µg/ml, 0.1µg/ml, 1.0µg/ml and 1.0µg/ml for ethambutol, isoniazid, rifampicin and streptomycin respectively.
5. Add appropriate reconstituted drug solutions into each of the corresponding labelled BACTEC MGIT 960 tubes.
6. Aseptically pipet 0.1 mL of the organism suspension into 10 mL of sterile saline to prepare the 1:100 Growth Control suspensions for day 1 and day 2 isolate. Mix the Growth Control suspension thoroughly.
7. Aseptically pipet 0.5 mL of the organism suspension from the original isolate into each of the FOUR remaining drug tubes (STR, INH, RIF,EMB).
8. Inoculate 0.5 ml of this suspension into the growth control-labelled tube from 1:100 diluted tube
9. Immediately recap the tube tightly and mix by inverting the tube several times.
10. Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
11. Be sure that the tubes are loaded according to the order specified for the AST set entry feature. Be sure that the caps are tightly closed
12. Open the desired MGIT 960 drawer and press the “tube enter” key.
13. The barcode scanner will light up
14. Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
15. Incubate MGIT tubes until the instrument flags them as positive
16. Check MGIT 960 daily for indicator lights flagging positive
17. Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer

Note: Second line drug susceptibility test using BACTEC MIGT 960 are the same procedure except drug and drug concentration different.

Annex 2: Protocol Genotypic DST or Line probe assay

Purpose

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

Principles

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

Amplification: Before amplification, amplification Mixes A (Taq polymerase, PCR buffer and nucleotides) and B(biotinylated primers, Mgcl 2 and are optimized for the PCR step of MTBDRplus test. The nucleotides acts as DNA precursors which will be used as building blocks during DNA polymerase (Hot Start Taq) elongate the DNA molecule.

Reveres Hybridization:

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

Procedures

Extraction:

1. Using a sterile disposable Pasteur pipette, transfer 1000µl of each thoroughly mixed liquid culture sample to labelled 1.5ml screw cap tube.
2. Proceed similarly to the procedure for decontaminated smear positive sediments
3. Load the 1.5ml screw cap tubes in a micro-centrifuge with aerosol-tight rotor.
4. Centrifuge for 15 minutes at 10,000 RCF or 10263RPM
5. Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC
6. Discard supernatant from each tube by use of a 1000µl adjustable pipette
7. Resuspend each pellet in 100µl Lysis Buffer (A-LYS)

8. Mix the contents of each tube by use of a sterile tip followedby thorough vortexing for at least 15 to 20 seconds
9. Incubate the tubes for 5 minutes at 95 °C in a thermoblock
10. Add 100 µl Neutralisation Buffer (A-NB) and vortex the samplefor 5 seconds
11. Load the tubes into a microcentrifuge and spin for 5 minutesat 13,000 x g
12. Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube
13. Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °C

Amplification and Detection:

1. Prepare a master mix containing AM-A and AM-B in a sterile screw cap tube (1.5ml) according to the number of samples and controls
2. Pipette 45 µl of the master mix to each labelled PCR tube
3. Add 5µl of DNA to corresponding master-mix PCR tubes in the PCR hood.
4. For first use, set up the thermal cycler to the correct amplification profiles according to the instruction manual.
5. Run the specific program (Ver.2-cul for samples from solid or liquid culture)
6. After the cycles are complete, proceed to the detection stage. If detection cannot be performed on same day, store PCR tubes with amplicons at 4°C for a maximum of 7days
7. Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total). Pre-warm RIN (rinse solution) and sterile distilled water to room temperature
8. Pre-warm TwinCubator to 45°C
9. Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided pen according to detection worksheet.
10. Pipette 20µl DEN (denaturing solution) to one end of each well of a clean tray to be used
11. Add 20µl of corresponding amplified DNA sample to the denaturing solution in each well, and mix well by pipetting up and down 5 times
12. Incubate for 5 minutes at room temperature

13. Carefully add 1 ml HYB (hybridization solution) to each well and in the opposite end to the DEN/DNA mixture. Use single tip for each well. Do not splash mixture or contaminate neighbouring well
14. Gently tilt to shake and homogenize solution. Do not splash mixtures
15. Add each labelled strip to each well with coloured marker facing up. If strips turn over, re-position them with a fresh pipette tip. Strips must be completely covered by hybridization solution
16. Place tray on Twincubator and press "START" to incubate for 30 minutes at 45°C. From this point, press right arrow on Twincubator once to advance steps in protocol.
17. When alarm goes off, press right arrow key to stop
18. Remove HYB carefully by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change tips or Pasteur pipettes between wells
19. Wipe off condensation that forms on Twincubator lid before every incubation step.
20. Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in Twincubator at 45°C. Press right arrow key to start.
21. When alarm goes off, press right arrow key. Completely remove STR as previously described for HYB removal.
22. Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips for 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.
23. Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on Twincubator
24. When alarm goes off, press right arrow to stop
25. Completely aspirate CON-D solution using Pasteur pipette
26. Add 1ml RIN per well. Press right arrow and incubate for 1 minute on Twincubator
27. When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20
28. Remove RIN completelyand wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator
29. When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.

30. Place on Twincubator under aluminum foil for a maximum of 10 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes
31. Wash twice for 1 minute with distilled water. Remove distilled water after each wash
32. Use forceps to transfer membrane strips to an absorbent paper and allow to air dry
33. Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water
34. Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol
35. Switch off the Twincubator after use
36. Use forceps to transfer strips to the GenoType MTBDRplus Results Sheet provided with the kit
37. Align the bands Conjugate Control (CC) and Amplification Control (AC) on each strip with the respective lines on the sheet
38. Attach the strips to the results sheet using clear adhesive tape
39. Determine the band positivity and positions on each strip using the reference reading chart of the kit and mark the results on the worksheet
40. In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.
41. If a positive result is obtained with the negative control, the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
42. In order for patient results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that M.tuberculosis complex is present in the sample
43. If CC is negative the conjugation or substrate reaction was unsuccessful either due to error in the procedure or due to problems with the reagent
44. If AC is positive, errors during extraction and amplification set-up and presence of amplification inhibitors in the specimen can be excluded
45. Signal of AC can be weak or even absent while results for other bands (TUB, rpoB, katG and inhA locus controls) may be positive. This might be due to competitive reactions

between AC and TUB, ropB, katG, inhA during amplification. In this case, the strip can be evaluated.

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

Note: Second line LPA test using method are the same procedure except master mix containing AM-A, AM-B and DNA strips different from first line LPA methods

Annex 3: Protocol for Genotype MTBC

Purpose

The GenoType MTBC test is a qualitative in vitro test from cultured material for the differentiation of the following species/strains belonging to the Mycobacterium tuberculosis complex (MTBC): M. tuberculosis/M. canettii, M. africanum, M. microti, M. bovis subsp. bovis, M. bovis subsp. caprae, and M. bovis BCG.

Principles of the Procedure

The GenoType MTBC test is based on the DNA•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from cultured material (solid/liquid medium; the necessary reagents are not provided), (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization. All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus the probes reliably discriminate the different sequences of the bacterial species. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

DNA Extraction

Bacteria grown on culture plates (e.g. Loewenstein-Jensen, Middlebrook) or in liquid medium (e.g. BACTEC, MB-Check) may be used as starting materiafor DNA extraction. The working area must be free from contaminating DNA.

The following quick protocol can be used for DNA extraction from cultured material:

1a. When using bacteria grown on solid medium, collect bacteria with an inoculation loop and suspend in approximately 300 µl of water (molecular biology grade).

1b. When using bacteria grown in liquid media, directly apply 1 ml. Pellet bacteria by spinning for 15 min in a standard table top centrifuge with an aerosol tight rotor in a class II safety cabinet at approximately 10,000 x g. Discard supernatant and resuspend bacteria in 100-300 µl of water (molecular biology grade) by vortexing.

2. Incubate bacteria from 1a or 1b for 20 min at 95°C in a water bath.

3. Incubate for 15 min in an ultrasonic bath.

4. Spin down for 5 min at full speed and directly use 5 µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, transfer supernatant to a new tube.

The assay on hand was also validated with the GenoLyse® kit (see chapter Ordering Information) which can alternatively be used for DNA extraction. For handling instructions, please refer to the instructions for use of the GenoLyse® kit.

The methods described above were used for performance evaluation of the GenoType MTBC. Until the present edition of the instructions on hand, the performance of the test has not been validated with other DNA extraction methods or sample materials.

Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. After thawing, stir AM-A and AM-B carefully. Pipette AM-A and AM-B only in a room free from contaminating DNA. The DNA sample should be added in a separate area.

Prepare for each sample:

- 10 µl AM-A (see Kit Component 2)
- 35 µl AM-B (see Kit Component 2)
- 5 µl DNA solution
- Final volume: 50 µl

Hybridization

When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the

hybridization protocol to be used. The following protocol describes the manual hybridization using a water bath or a TwinCubator.

Preparation

Prewarm shaking water bath to 45°C (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on TwinCubator. Rewarms solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1. Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.
2. Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
3. Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color. Take care not to spill solution into the neighboring wells.
4. Place a strip in each well. The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.
5. Place tray in shaking water bath/TwinCubator and incubate for 30 minutes at 45°C. Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.

6. Completely aspirate Hybridization Buffer. For example, use a Pasteur pipette connected to a vacuum pump.
7. Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator.
8. Work at room temperature from this step forward.
9. Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).
10. Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator.
11. Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water
12. Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.
13. Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.
14. Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is provided with the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and UC with the respective lines on the sheet. Note down positive signals in the last but one column, determine species with the help of the interpretation chart and enter name of the identified species in the last column. The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and UC of the strip as well. Each strip has a total of 13 reaction zones

Troubleshooting

Overall weak or no signals (including Conjugate Control zone)

- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used. Repeat reverse hybridization.

Weak or no signals except for Conjugate Control zone

- Quality of extracted DNA does not allow an efficient amplification. Repeat extraction.
- Amplification Mixes (AM-A and AM-B) were interchanged, added in wrong amounts, or not stirred properly. Prepare a new master mix and repeat test.
- Incubation temperature too high. Repeat reverse hybridization.
- The extracted bacterial species cannot be detected by the Universal Control. Use alternative identification method.

No homogeneous staining

- Strips were not completely immersed during incubation steps.
- Tray was not shaken properly. Repeat reverse hybridization.

High background color

- CON-C and/or SUB-C used too concentrated.
- Washing steps were not performed with the necessary care.
- Wash solutions too cold. Repeat reverse hybridization.

Unexpected result

- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly prewarmed or mixed.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer. Repeat reverse hybridization.
- Contamination of extracted DNA with previously extracted and/or amplified DNA. Repeat extraction.
- Contamination of amplification reagents. In this case, a negative control sample shows additional bands besides CC. Repeat amplification using fresh reagents.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands.
- No pure culture as starting material. Re-culture in order to exclude contamination.

- Error during DNA extraction. Repeat extraction.
- The bacterial species present in the sample cannot be detected with this test. Apply additional detection methods.

Annex 4: Protocol for Spoligotyping

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

Spoligotyping Principle

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

Procedure

1. Always include chromosomal DNA of Mycobacterium tuberculosis strain H37Rv and Mycobacterium bovis as positive controls and primary buffer use as a negative control.

A. **Prepare the Master Mix reaction:**

Reagent	1	50
HotStarTaq Master Mix	12.5ul	625
Primer (DRa)	2ul	100
Primer (DRb)	2ul	100
Molecular grade Water	3.5ul	175

a. Add 5ul of your sample
b. Select the program from the PCR machine
c. Make sure the volume is 25ul
d. Run the PCR
e. Store at 2-8 ^{O}C till detection

Reagent preparation

Reagent used for hybridization

Primary buffer (2XSSPE/ 0.1SDS)

250ml/round=25ml 20XSSPE + 222.5ml distill water + 2.5ml 10%SDS

- Used to dilute PCR product –allocate 10ml and store at room temperature
- Used to pre-warm the membrane-the remaining 240ml will be equilibrate at **60^{O}C**

Secondary buffer (2XSSPE/ 0.5SDS)

1000ml/round=100ml 20XSSPE + 850ml distill water + 50ml 10%SDS

- Used to remove unbound PCR product and residual POD
- Split the buffer into two: Equilibrate 500ml at **60^{O}C** and 500ml at **42 ^{O}C**

2XSSPE

500ml/round= 50ml 20XSSPE + 450ml distill water

- Used to remove excess SDS: store at **room temperature**

1. Reagent used for membrane washing and storage

1%SDS

500ml/round= 50ml 10%SDS + 450ml distill water

- Used to remove the PCR product: Equilibrate at **60^{O}C**

20mM EDTA

500ml/round= 20ml of 0.5EDTA + 480ml of distill water

- Used to wash and store the membrane: Store at room temperature

Hybridization with PCR product and detection

1. All buffers should be pre-warmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):
   - Primary buffer (2×SSPE/0.5 % SDS, at 60 °C)
   - Primary buffer (2×SSPE/0.5 % SDS, at 42 °C)
   - Seconder buffer (2×SSPE/0.1 % SDS, at 42 °C)

- 2×SSPE, room temperature.

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

Detection procedure

1. Prepare ECL by adding 10ml of solution 1 and 10ml of solution 2
2. Use specific container marked as “ECL” and add the ECL solution to the membrane
3. Immerse for 1-2min and ensure the membrane is completely covered in the solution
4. Use cassette and prepare used film rapped with plastic (to support the membrane) and place the membrane on it
5. Cover with plastic sheet and avoid bubble
6. **Work in dark room**, put a new film on top, close the cassette, and expose for 10-20min (depending on the intensity of black square)
7. Ensure film developer (100ml), water and fixer(100ml) is ready
8. Turn on the red light, remove the film and place into the developer. Ensure it is entirely submerged

9. Tilt gently utile an image of black square is visible
10. Rinse with distilled water for 5-10sec
11. Immerse into trays containing fixer, ensure it is entirely submerged. Agitate gently for 1-2min
12. Rinse with water for at least 2min and allow to dry
13. Label the film with round number and DRS number
14. Discard the developer but transfer the fixer into labeled dark bottle. The fixer can be used for 5 times (with maximum one month)

Trouble shooting

1. Spoligotyping demands that the procedures are followed in an accurate and careful way. This improve our final result
2. However, the list below enhance our result in case of poor spoligopattern
3. In case of weak spoligopattern, the signal can be enhanced by longer the exposure time of the film to the membrane i.e. 30-40min
4. In case of strong spoligopattern, the signal can be improved by shorter the exposure time of the film to the membrane i.e. 10min
5. In case of patchy or dark shadows spoligopattern, wash the membrane **two times** in 250ml of 2XSSPE at room temperature for 5min with shaking and repeat the whole detection procedure

A. Regeneration of the membrane (Membrane washing procedure)

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

1. Unwrap the membrane and wash **two times** with 250ml of 1% SDS (stored at 60 ^{O}C) for 30min at 80 ^{O}C with shaking
2. After the second wash leave at room temperature for 5min to cool before discarding the liquid
3. Wash for 5min in 250ml of 20mM EDTA
4. Discard the liquid & add another 250ml of 20mM EDTA & store the membrane at 2-8 ^{O}C.

Declaration

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

M.Sc. candidate: Getu Diriba (B.Sc.)

Signature_____

Date of submission_____

This thesis has been submitted with our approval as advisers.

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