ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Investigation of Strain Diversity and Trend of Drug Resistance Pattern of the Clinical isolates from Tuberculosis lymphadenitis Patients in Two different Cities of Ethiopia

BY: Sami Sebri

Advisors: Mr. Kassu Desta (MSc, assistant prof)
Dr. Melese Hailu (MSc, PhD)
Mr. Shambel Araya (BSc, MSc)

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

JUNE 25, 2023
ADDIS ABABA, ETHIOPIA

I. ACKNOWLEDGMENT

First, I would like to acknowledge Addis Ababa University for the opportunity of the graduate study it is delivering in which I am perusing my master's degree and AHRI for sponsoring my research. Furthermore, I am highly indebted to my advisors, Mr Kassu Desta, Mr.Shambel Araya, Mr Melaku Tilahun, Dr Kidist Bobosha and Mr Abay Atnafu Dr. Melese Hailu for their unreserved support during the entire stages of this work without which it would not have been realized. And my heartily felt thanks go to the Almighty God.

II. TABLE OF CONTENT

III. LIST OF TABLES

IV. LIST OF FIGURES

V. LIST OF ABBREVIATION

CDC Center for Disease Control

DST Drug Susceptibility Test

EPTB Extra-pulmonary Tuberculosis

FNAC Fine- needle aspiration cytology

INH Isoniazid

LPA Line Probe Assay

MGIT Mycobacteria Growth Indicator Tube

MIRU-VNTR Mycobacterial interspersed repetitive unit-Variable number tandem repeats

MTBC Mycobacterium Tuberculosis Complex

NAAT Nucleic Acid Amplification Test

PTB Pulmonary Tuberculosis

RIF Rifampin

TB Tuberculosis

TBLN Tuberculosis lymphadenitis

WHO World Health Organization

Abstracts

Background: Identification of *Mycobacterium tuberculosis* strain have a significant contribution to the understanding of genetic diversity in a particular area. The most commonly occurring type of extra-pulmonary tuberculosis is TB Lymphadenitis. Tuberculosis (TB) is a complicated and chronic infectious disease and severely affect the organs. Investigating and understanding the association between Molecular characteristics and drug susceptibility of (MTBC) strains are important to comprehend the transmission dynamics and drug resistance pattern.

Objective: To investigate strain diversity and trends of drug resistance pattern of the clinical isolates from Tuberculosis lymphadenitis (TBLN) patients in two different cities of Ethiopia.

Methods: Institution-based cross-sectional study was conducted from 52 previously stored isolates collected from TBLN patients in 2019 from Ambo and from ALERT comprehensive hospital in 2021. Sub-culture using LJ, Line Probe Assay, and Spoligotyping were performed from the stored isolates. Descriptive statistics were used to present frequency of data. A statistical analysis was done using SPSS Version 26. A test with a P value of < 0.05 was considered statistically significant.

Results: A total of 52 culture positive confirmed M. tuberculosis isolates have been used for drug sensitivity testing and strain identification. Out of the 52 spoligotype patterns (strains), 29 isolates matched the preexisting patterns in the SITVIT2 database, the rest 23 found to be new or orphan strains according to SITVIT2 database. Classification of MTBC strains from both cities showed the occurrence of the following predominant lineages, Euro-American (40/52 76.9%), followed by Indo-Oceanic (2/52; 3.8%), and East-African (9/52;17.30%). isolates in two different cities were investigated, isolates from ambo collected in 2019 identified to be sensitive for all major anti-TB drugs. However, culture positive samples collected in 2021 from Addis Ababa have shown resistant strain for both anti TB-drugs, 4 (15%), 3(11.5%) of them have been resistance to both RIF and INH, and the rest 1 isolate were Mono- resistant to INH 1 (3.8%).

Conclusion: This study determines the association of drug resistant TB with corresponding lineage and molecular epidemiology in patients with tuberculous lymphadenitis. Lineage 3 and Lineage 4 represented to be most prevalent lineage of *M. tuberculosis with* high clustering rates of SIT 125, SIT149 and SIT 53, more over these lineages are associated with most of drug resistance *M. tuberculosis.*

Keywords: Extra-Pulmonary tuberculosis, Line probe assay, and Drug resistance**.**

1. Introduction

1.1 Background

Tuberculosis (TB) persists one of a significant global public health problem. Furthermore one-third of the world's population is estimated to be infected with mycobacterium tuberculosis. Pulmonary TB (PTB) and extra-pulmonary TB (EPTB) are the two types of TB, based on the anatomical sites where the disease is manifested (1). According to the global estimation report extra-pulmonary TB (EPTB) ranges from 17% to 24% of all TB cases (2).

EPTB is a collective term of MTB infection of organs such as Lymph node, cutaneous tissue, Gastero intestinal Tract (GIT) system, and bones (3)**.** The most commonly occurring type of extra-pulmonary tuberculosis is TB Lymphadenitis. Tuberculosis (TB) is a complicated and chronic infectious disease and severely affect the lung, the lymph node, and other parts of the body. Pulmonary tuberculosis (PTB) and extra-pulmonary tuberculosis (EPTB) remains significant public health problems in Ethiopia. Extra-pulmonary tuberculosis can be diagnosed clinically as well as bacteriologically confirmed in organs besides the lungs. Molecular identification of extra-pulmonary TB have a significant role in reducing TB-related morbidity and the complications that causes lifelong sequelae, and disabilities (4, 5). Extra-pulmonary TB has higher incidence rate in Ethiopia since the 1990s. TB lymphadenitis accounts for about 80% of all extra-pulmonary cases (3).

Various molecular identification methods have been adopted for the investigation of MTB strain. Spoligotyping is commonly used to characterize strain and helps to reduce the transmission by tracing the community/area infected by that particular strain (6). Spoligotyping mainly identifies genetic diversity in the direct repeat (DR) region of MTB by the formation of patterns particular polymorphism presentation in the DR locus of MTB, used to investigate various lineages and sub-lineages of MTB complex and considered as a gold standard for linage identification (7).

Among the eight well-known lineage that are identified worldwide, Ethiopia has a unique and specific lineage /lineage 7/ that has been confirmed by causing human disease both PTB and EPTB. Lineage 7 is believed to be an evolutionary strain that has been identified in the Horn of Africa especially in Ethiopia and intermediate between the ancient and modern lineages of Mycobacterium tuberculosis (8). There is a variance between MTBC lineages regarding

inflammatory response, immunogenicity, virulence depends on host genotype and geographical location (9).

Investigating and Understanding the association between Molecular characteristics and drug susceptibility of Mycobacterium Tuberculosis Complex (MTBC) strains are important to comprehend the transmission dynamics and drug resistance pattern Different types of molecular typing techniques, based on deoxyribonucleic acid (DNA) fingerprints have developed and used to diagnose and characterize MTBC / been (Spoligotyping, IS6110-RFLP, Polymorphic GCrich repetitive sequences (PGRS)-RFLP, Mycobacterial interspersed repetitive unit (MIRU)-VNTR)/ (10).

Recently there are some studies reporting the drug resistance pattern of MTB from Tubercular lymphadenitis patients where most of them narrates an increased proportion of Mono/MDR TB among TBLN patients. However, there is paucity of data showing the trend in the development of drug resistant TB different time points and the comparison between different setting in Ethiopia. On the other hand, the strain diversity in different time periods and different setting in Ethiopia was seldom studied.

Therefore, this study aimed to investigate the strain diversity and the trend of drug resistance pattern from clinical isolates collected from TBLN patients in Ambo and Addis Ababa.

1.2 Statement of the problem

Tuberculosis lymphadenitis (TBLN) stated as highly emergence of most common MTB strains and causes a serious life-threatening complication and disabilities than other extra pulmonary tuberculosis species (11). Moreover, it is the most frequently occurring form of EPTB as well as drug-resistant EPTB (12).

Extra pulmonary TB remains a vital public health problem in developing countries due to challenges associated with diagnosis. According to recent evidence the TBLN burden is growing with alarming rate and halting of new cases globally is nevertheless challenging. Therefore, prevention of TBLN and other forms of TB required to be a public health urgency(13). WHO estimation report indicates, regarding the number of EPTB patients, with high TBLN diagnosis, Ethiopia ranked 3rd globally following India and China, which shows high burden of Tuberculosis lymphadenitis in different region of Ethiopia (14).

Most nations with a high TB-burden have resistance mutation to INH and RIF, this mutations occurs between codons 526 and 531.(15). Drug resistant TB, both mono/MDR persists a major public health concern in many parts of the world. Moreover, five hundred thousand people developed MDR/RIF resistant TB in 2019 worldwide (16). According to WHO report the outgrowth of drug-resistant *MTB* causes difficulties on global TB control program(17). Regarding this investigations conducted in Ethiopia, shows the magnitude of resistance EPTB, 7.0% for INH, 6% for RIF and 4% for MDR-TB.(18).

MTB modifies gene expression to adapt to the environment, which might affect the structure and function of the organism and make it more challenging for the immune system to eradicate it. (14)**.** EPTB is more difficult to diagnose and treat because the bacteria that cause TB can become resistant to antibiotics and the symptoms are often not specific(8)**.** Tuberculosis lymphadenitis thought to be the most prevalent indication of EPTB in most setting and considered to be triggered by diffuse of bacteria by heamtogenous route from first exposure lung to signify a disseminated form of TB disease(19)**.**

Global estimation in 2019 shows, Rifampicin-resistant tuberculosis (RR-TB) affects roughly 500,000 people worldwide, and 82% of those cases are MDR-TB. (20). Treatment of Multi drug resistance TB costs much, difficult to treat, it has a high risk of relapsing, and remains challenge for TB control program due to unsuccessful therapy and more often fatal. MDR-TB occurs due to specific gene mutation of Tuberculosis (21). Despite the effort in early case detection, MDR-TB remains difficult to treat and prevent transmission among both new diagnosed and mostly previously treated patients due to shortage of drug susceptibility tests(22).

Studies indicate that frequent infected sites is thought to be lymph node, so that most of EPTB cases are referred to TB lymphadenitis followed by TB peritonitis (2). Conventional method diagnosis is poor on detecting EPTB and the longer time required, and also the difficulty of sampling and the paucibacillary nature of samples which makes it challenging(23) Furthermore, genotyping method studies have not been well conducted in Ethiopia such as Spoligotyping profile MTBC strains that are used for comprehending the molecular epidemiology of TB which is essential for regional disease control and investigate the association between particular strains and an outbreak(3).

Since most of the population in developing countries (70%) uses below standard health facilities, hence studying through genetic diversity and genetic polymorphism among Drug-resistant tuberculosis is indicated to be vital (23). Although, most common factors that have high contribution to genetic diversity is observed to be the deletion of large sequence polymorphisms (LSP), duplication, and insertion sequence IS*6110* since it's highly polymorphic that mostly takes place in single nucleotide polymorphisms (SNP) and is responsible for formations of different types of lineage according to studies (24).

Regarding to the distribution of MTBC lineages in various region of the country. The degree of disease manifestation, the form of treatment resistance, and host adaptation are all significantly impacted by its enormous regional diversity**(8).**

The study aims to investigate the strain diversity and the trends of drug resistance pattern in clinical isolates from TBLN patients and provides information on the transmission dynamics of strain and have implications for the development of new diagnostics, drugs, and vaccines, to reduce a major challenge to TB control strategy worldwide.

1.3 Significance of the study

This study contributes significantly to the understanding of the molecular Epidemiology of the specific strain in the study area provides information regarding a certain anti-TB drug-resistant strain's dominance and the association of a particular strain with multi drug resistant TB. Identifying and understanding the genetic diversity of MTB strains of descent or lineage of MTB strains are relevant to manage a particular circulating MTB homogenous strain responsible for an outbreak or control transmission of infectious strain in the community. The genotyping data are valuable as an input to map the population structure of MTBC in both study areas and They are appropriate for supporting programmatic management and drug-resistant TB surveillance.

2. Literature review

The proportion of EPTB in some parts of the world is lower than that of the other developed countries such as Italy (32%) and Australia (39%) and the most common manifestation was TB lymphadenitis (35.3%), which was followed by TB peritonitis (12.05%) and tubercular pleural effusion (6.7%) according to this finding.(2).

Predominant MTB strain lineage that have association with MDR-TB are observed to be T lineages and CAS lineages followed by some other six lineages, Haarlem, F33, F36 and LAM9 according to Spoligotyping pattern which has a major role in identifying MTB strain lineages conducted in Addis Ababa Ethiopia (17). Furthermore, There are two common ways of detections for Spoligotyping that can identify MTBC lineages pattern in a particular community in cluster formation and orphans, although, Spoligotyping patterns indicates most common isolates that account about 86.5% were found to be orphans (6). likewise, MTB strain mutations in the inhA and katG genes can be detected by molecular technologies as LPA, which has a (5%) greater detection ability(15).

Other study conducted in South Africa indicates the variation of strain diversity and drug sensitivity, Among 79 MDR TB isolates there are three major strains were identified (ST34 S/Québec family, ST60 LAM4/KZN, and ST53 T1 family), this is a Spoligotyping pattern that is highly linked with drug-resistance TB and described as a wide range of strains that are identified among drug-susceptible TB only a few have diversified genetic makeup among drug resistance TB (25). Furthermore SIT 149 of T3- ETH, SIT21 of CAS1-KILI, and SIT25 of CAS1-DELHI has a strong link to MDR-TB, as well as sub-lineages like CAS1-KILI, CAS1-DELHI and T3-ETH are highly related with MDR-TB (17).

According to research finding Spoligotyping patterns were done to group similar Predominance families, T1, family33, H37Rv, and CAS that accounts for 71.4% of the total teste samples, the rest have (15/104) and 11.5% (12/104), respectively (25). Since Spoligotyping cluster formation mostly indicates a recent infection, most commonly Modern lineages CAS and Beijing are considered to be highly mutated strain linage that has a significant association with drug-resistant tuberculosis (7). also In some parts of Ethiopia, it's believed to be a particular lineage has a higher association with MDR-TB such as Haarlem than other types of lineages (17).

A study conducted in India reviews among 135 MDR isolates CAS and EAI accounts for only 27% and 8% respectively, the rest of resistance isolates were reported as Beijing, /SIT1 sub-lineage/ which has a significant association with MDR-TB (7). Furthermore, High distribution, immunogenicity, and virulence of EPTB might have an association with types of MTB strains according to a study; also, the site of infection is mostly linked with a specific strain. Around 42% of 59 EPTB isolates from tuberculosis lymphadenitis were belonging to the Harlem sub lineage (26).

A particular study was conducted in the community on culture positive isolates, which indicates the association between the current MTBC lineages and drug-resistant TB. This study indicates that lineage three /L3/ and lineage four /L4/ dominates among drug-resistance TB strains and only 0.4% TB drug-resistant was reported for /Ethiopia-specific lineage 7/ (8). Most of the studies conducted referring to MTB lineages indicate, The two major lineages L4 and L3 as the predominant lineages(3).

Despite our limited understanding of the molecular epidemiology of drug-resistant TB, a study found that among the eight major lineages, Ethiopia-specific /lineage 7/ has higher prevalence of causing lymph node TB illness.(8). However, various studies indicate that lineage four (L4) and lineage three (L3) is thought to be the most common lineage in Ethiopia depending on geographical locations and accounts for about 71% and 47% respectively and have an equal distribution for PTB and TBLN according to a study conducted in northern sites of Gondar and Woldiya (27).

Tuberculosis lymphadenitis samples were further checked following culture and GenoType MTBDRplus VER 2.0 Spoligotyping, for identifying genetic mutation and lineages. Most of the samples were grouped with nine clusters the others are believed to be an “Orphan” strain. However, various types of strain were identified / Lineage 1 up to 7/ lineage 3 and lineage 4 were predominantly identified, the other lineages were the minimum prevalent lineage (16). Additionally, Strain sub-lineages were also identified in some other study, such as CAS sub-lineage that accounts about 34% of the L3 sub-lineage and T1, T3-ETH and Haarlem accounts about 40% of the total of sub-lineage four (L4) including family sub-lineages LAM (LAM, LAM3, and LAM5), also the subsequent sub-lineages differ according to geographical location (3).

Different studies conducted points out, among all TBLN isolates that have been tested with GenoType MTBDRplus VER 2.0 particularly in northern and southern parts of Ethiopia/ for

specific genetic mutation, indicates most of a genetic mutation occurs in a particular codon region/S531L/, predominantly related with RIF and INH resistance, which has Specific mutated genes such as *rpoB* gene, katG, inhA promoter region that accounts around (2.2%) (4.4%), (2.3%) respectively (16). On the second study, similar method / GenoType MTBDRplus (v1.0)/ has been used to identify both PTB and TBLN for anti-TB drug /RMP or INH/ resistance and identified to be 14 isolates are resistance among 160 total samples. In addition, Further Spoligotyping techniques have been used to gather information on the association of lineages with anti-TB drug resistance and found to be "lineage four" has a higher proportion of anti-TB drug resistance followed by "lineage two". Furthermore, most prevalent Spoligotyping lineage cluster in Ethiopia was observed to be modern lineage SIT 149 (T3-ETH, ETH-3) recently referred to as lineage four /L4/ (28).

The major M. tuberculosis Spoligotyping strain cluster that causes TBLN is observed to be the SIT149 sub-lineage of T3-ETH, this is frequently identified strain of M. Tuberculosis. The second prevalent strains among TBLN patients are T1, CAS/DELHI, T3 with sub-lineages SIT53, SIT26, SIT37 respectively (29). Similar study indicated that M. tuberculosis confirmation was done by PCR-based typing and the pattern of strains was checked by Spoligotyping comparing with current databases. According to this study in some parts of Ethiopia T, CAS, and H, were predominant strains and show less association with anti TB drug resistance compared to the Beijing lineage which is highly prone to anti-TB-drug resistance (30).

This study aims to provide information on strain diversity and Drug-resistant tuberculosis patterns of the stored clinical isolates from AHRI's TB laboratory biorepository.

3. Objective

3.1 General objective

- To investigate strain diversity and the trend in drug resistance pattern of the clinical isolates from tuberculosis lymphadenitis patients in two different cities Ambo and Addis Ababa Ethiopia in 2021.

3.2 Specific objective

- To identify MTB linages and strain diversity of MTB among clinical isolates from TB lymphadenitis in Ambo surroundings and ALERT hospital Addis Ababa Ethiopia in 2021.
- To investigate the association between circulating MTBC lineages and drug resistance pattern of the clinical isolates from TB lymphadenitis in Ambo surroundings and ALERT hospital Addis Ababa Ethiopia in 2021.
- To investigate the trends of drug resistant pattern through time among clinical isolates from TB lymphadenitis patients in Ambo surroundings and ALERT hospital Addis Ababa Ethiopia in 2021.

4. Method and materials

4.1 Study area and period

In this study, a total sample size of 52 culture positive archived isolates which is previously collected for dissimilar studies, were used from two different cities, Ambo western part of Ethiopia in 2019, and Addis Ababa ALERT hospital in 2021. FNA samples collected from Both study areas

4.2 Study design

An institution based cross-sectional study was conducted.

4.3 Population

4.3.1 Source population

All clinical isolates collected from EPTB patients and stored at AHRI TB laboratory repositories.

4.3.2 Study population

All clinical isolates collected from EPTB patients and stored at AHRI TB laboratory repositories.

4.4 Eligibility criteria

4.4.1 Inclusion criteria

All recoverable clinical isolates collected from TBLN patients in Ambo and Addis Ababa town, Ethiopia.

4.4.2 Exclusion criteria

Non recoverable isolates and contaminated isolates were excluded from the study.

4.5 Study variables

4.5.1 Dependent variable

culture positive isolates, Drug-resistance MTB strain, MTB lineages/sub-lineages, MTB families,

4.5.2 Independent variable

Socio-demographic: - Age, Sex, Educational status, income, marital status,

4.6 Measurement and data collection

4.6.1 Sample size determination

Some basic assumptions of sample size determination

$Z\alpha/2^2$–(level of confidence) = 0.05 (95%).

P (proportion) = 0.083(18)

d2 (the degree of precision) = 12% of P = 0.83*0.12 = 9.96 ≈ 10%

n (sample size) =

Non response rate = 10%

$$n=\frac{1.96^2.0.083(1-0.083)}{(0.1)^2}$$

n = 54

Total sample size = 64

4.6.2 Data collection procedure

Socio demographic and other relevant data was retrieved from AHRI's TB laboratory archive and the clinical isolates were collected according to their culture positivity pattern from an archive of previous studies. Following the selection of culture positive samples, isolates were collected from AHRI isolate repositories.

4.6.3 Recovery of isolates using LJ media

Isolates were Thawed. 0.1 mL of the sample was inoculated into standard LJ medium. evaluated twice weekly for 6 weeks at 37°C in a slant position, then at 8 weeks before being discarded(31, 32).

4.6.4 Region of Difference 9 deletion typing

PCR-based region of difference (RD9) and RD4 techniques were used. 10 µl of Hot-StarTaqMaster Mix, 7. µl of molecular grade water, 03 µl of primers, and 2 µl of DNA MTtemplate. M. bovis and TB H37Rv served as positive controls, while Qiagen water served as a negative control. The program called for heating and amplification of the combination. Using the Agarose Gel Electrophoresis System, 100 base-pair DNA was electrophoresed on the gel. using a Multi-Image Light Cabinet to visualize. If 575 bp was either M. bovis or M. africanum and 396 bp was M. Tuberculosis(33).

4.6.5 Genotype MTBDRPlus

Genotype MTBDR*plus* VER 2.0 assays for the identification of mutations in the rpoB gene for rifampicin resistance, the katG gene for high-level isoniazid resistance, and the inhA gene for low. Heat-killed mycobacterial culture and DNA was extracted by GenoLyse. Then amplified PCR. Amplicons were hybridized with oligonucleotide probes. detect color band present or absent, wild type for INH and common mutation for *kat*G and *inh*A. susceptible all WT probes were detected and MUT probes were not detected and resistance for vice versa (34). H37Rv strain for positive and molecular grade water for negative control (33).

4.6.6 Spoligotyping

Spoligotyping was carried out based on the spoligo standard method. The direct repeat (DR) region was amplified by PCR using oligonucleotide primers DRa and DRb. M. bovis SB 1176 and H37Rv positive control and Qiagen water as a negative contro were used. 25 µL rxn mix, 2 µL primers, 5 -µL heat-killed cell suspension, and 3.5 µL distilled water. The mix amplified by PCR according to the program. hybridization to a set of 43 immobilized oligonucleotides. The membrane was washed twice for 10min in 2× SSPE and 0.5% SDS at 60°C and streptavidin-peroxidase for 45–60 min at 42°C. Then membrane washing similarly. After the hybridizing, DNA detected by exposure to X-ray-film. The patterns converted into binary and octal formats entered in to (SIT) (33). Lineages and sublineages were designated based on SPOTCLUST http://tbinsight.cs.rpi.edu/run_spotclust.html, (29).

4.6.7 Work flow

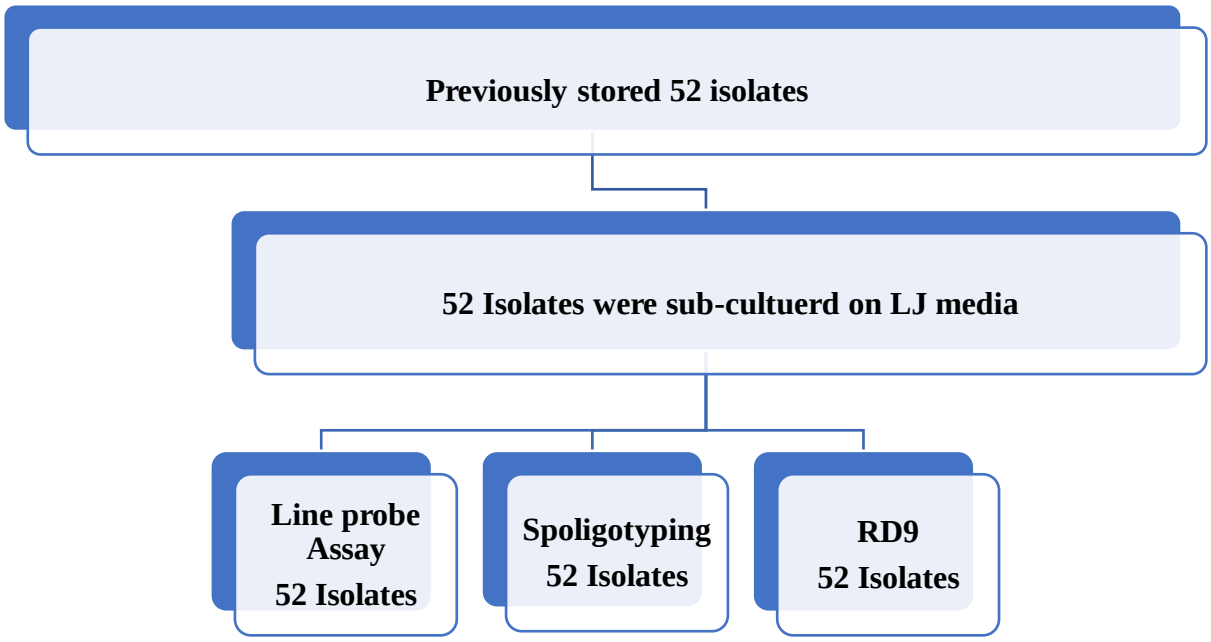

Figure 1. Work flow

4.7 Data quality assurance

Archived sample was used. Quality of the sample was measured according to different guidelines as well as the institutes policy. Storage condition, specimen stability, sample volume and adequate information was checked before proceeding for further techniques. Additional data / personal information's/ was collected from the institute archives.

4.8 Data analysis and interpretation

All demographic and laboratory data was entered, cheeked and analyzed using SPSS version 26.0. Descriptive analysis of the variables was done so as to obtain frequency distribution of all variables. The chi square test was used to measure the level of association and statistical significance was declared at p-value <0.05.

4.9 Ethical consideration

The proposal was submitted for approval by research Ethical review committee of AHRI/ALERT. Permission was taken from Ethical review committee of AHRI/ALERT. The data was used solely for research purposes.

4.10 Operational definition

Extra pulmonary TB: Is an infection by MTB which affects tissues and organs outside the pulmonary parenchyma.

Drug Resistance TB: Resistance of MTB to at least isoniazid and rifampicin the cornerstone medicine for the treatment of TB.

TB Lymphadenopathy: A chronic specific granulomatous inflammation of the lymph node with caseation of necrosis caused by infection with MTB.

5. RESULT

5.1 Socio-demographic characteristic

A total of 64 culture positive Tuberculous lymphadenitis isolates were sub-cultured in this study. Among them 52 isolates have been grown successfully on LJ media and stored using freezing media. All 52 isolates were enrolled in this study for molecular characterization and further testing of Drug sensitivity testing. The socio demographic and clinical characteristics of culture positive isolates were collected retrospectively from previously archived questionnaire. Among the total of 52 culture positive participants, 25 (48.07%) were males and 37 (71.15%) were females. The patients' mean age was 33 (SD+13.9) years with a range of 18–70 years. Most of the participants [30, (57.6%)] were illiterate, Among the participants 38.4% were house wives, and 23.07% of the participants were from rural part of the town as shown in (table 1).

Table 1. Socio demographic characteristic of the study participant

Variables		**Frequency (%)**	**Culture**	
			Positive	**P-Value**
Age	18-24	32.6	17	
	25-34	25	16	
	35-44	17.3	12	
	>44	13.46	7	0.974
Sex	Male	38.46	20	
	Female	61.5	32	0.77
Residence	Ruler	23.07	12	
	Urban	76.9	40	0.538
Marital status	Single	42.3	22	
	Married	57.6	30	0.686
Education	Illiterate	57.6	30	

	Elementary	26.9	14	
	High school	15.38	8	0.908
	College and above	0	0	
Occupation	Unemployed	15.8	8	
	Civil servant	0	0	
	Student	25	13	
	House wife	38.4	20	
	Merchant	3.8	2	
	Farmer	15.38	8	
	Driver	1.9	1	0.968

5.2 Clinical characteristics

Among the entire culture positive study participant only 2 (3.8%) had hemoptysis. Participants who consume raw milk were 26(50%). Raw meat consumption was observed in 42(80.7%) (table 2).

Table 2. Clinical characteristics of the study participants

Variables		**Frequency (%)**	**Culture positive**	**P value**
Hemoptysis	**Yes**	**3.8**	2	0.974
	No	**96.1**	50	
Fever	**Yes**	**67.3**	35	0.849
	No	**32.6**	17	
Chest pain	**Yes**	**38.46**	20	0.131
	No	**61.5**	32	
TB Patient in the house	**Yes**	**9.61**	5	0.782
	No	**90.3**	47	
	Raw	**50**	26	0.851

Milk consumption	**Boiled**	**32.6**	17	
	Both	**9.61**	5	
	I don't drink	**7.6**	4	
Row meat consumption	**Yes**	**80.7**	42	0.782
	No	**19.2**	10	
History of Hospital admission	**Yes**	**5.76**	3	0.167
	No	**94.2**	49	
Imprisonment	**Yes**	**1.9**	1	0.475
	No	**98**	51	
Alcohol consumption	**Sometimes**	**36.5**	19	0.529
	Always	**9.6**	5	
	never drink	**53.8**	28	
HIV Status	**Positive**	**1.9**	1	0.551
	Negative	**17.3**	9	
	Unknown	**80.7**	42	

5.3 RD9 deletion

Strain typing using RD9 deletion typing was performed for all culture positive isolates. All strain belongs to M. tuberculosis (Table 3).

Table 3. RD9 Deletion

		Culture	Total
		Positive	
RD9 deletion typing	Present	52	52
	Absent	0	0

5.4 Drug sensitivity test result

The MTBDRplus assay was performed on isolates from two cities and Culture positive samples were detected to be resistance for major Anti-TB drugs. Furthermore, among 52 culture positive Isolates, 4 (7.6%) of the isolates were found to be Resistant to anti-TB drugs, 3 (5.7%) of them have been resistance to both RIF and INH, and the rest 1(1.9%) isolate were Mono- resistant to INH shown in (Table 4,5,6).

Table 4. Drug sensetivity test result

		Culture positive isolates Ambo	Culture positive isolates Addis Ababa
INH	Sensitive	26	22
	Resistant	0	4
RIF	Sensitive	26	23
	Resistant	0	3
MDR	YES	0	3
	NO	26	23

Table 5. Drug resistance patterns

Resistance	Frequency	Percentage (%)
Any drug resistant	4	7.6%
INH	4	7.6%
RIF	3	5.7%
Mono-drug Resistant	1	1.9%
INH	1	1.9%
RIF	0	0
Two or more drug resistance	3	5.7%
INH + RIF	3	5.7%

52 culture-positive individuals' drug resistance pattern to first-line Anti-Tuberculosis medications of Mycobacterium TB from Ambo and Addis Ababa

Abbreviations: INH, isoniazid; RIF, rifampicin.

Table 6. Drug resistance pattern and Mutation

Gene	No of resistance	Mutation Pattern	Amino Acid change	Result
rpoB	3	ΔWT8, MUT3	S531L	RIF r
katG	4	ΔWT, MUT1	S315T1	INH r

Rifampicin and isoniazid-related drug resistance pattern and mutations with the genotype MTBDRplus VER 2.0

Abbreviations: Δ, deletion; INH, isoniazid; INH r, isoniazid resistant; MUT, mutant; RIF, rifampicin; RIF r, rifampicin resistant; WT, wild-type.

5.5 Spoligotyping and Mycobacterium lineages

MTBC species were isolated from 52 TBLN patients from both cities Ambo and Addis Ababa. The isolates were identified at strain and lineage levels. A total of 52 MTBC isolates were spoligotyped from both cities Ambo and Addis Ababa, and 29 (55%) of different spoligotype patterns (strains) were identified. The dominant strains were SIT53, SIT149, and SIT336, each consisting of 4 (27%), 4 (25%) and 3 (18.75%) isolates respectively. These strains contributed 55% (16/29) of all isolates with known spoligotype patterns. Further identification of dominant strain, from Addis Ababa SIT37 consists 2(7%) of the isolates. Out of the 52 spoligotype patterns (strains), 29 isolates matched the preexisting patterns in the SITVIT2 database, the rest 23 found to be new or orphan strains according to SITVIT2 database. Classification of MTBC strains from both cities showed the occurrence of the following lineages, the predominant lineage from Ambo were, Euro-American (23/26 88.4%), followed by Indo-Oceanic (2/26; 7.6%), and East-African (1/26; 3.8%). Similar identification from Addis Ababa major lineage were (7/26; 65.3%) and East African Indian (8/26; 30.7%). Sub-lineages from Ambo city isolates, T1, Haarlem and T3 are dominant strains in current study which accounts 8(30.7%), 5(19.2%), and 4(15.3%) respectively, the rest of strains CAS, Haarlem1, and X1 have low prevalence in that of particular area. The predominant sub-lineages from Addis Ababa isolates, CAS1-Delhi, T3-ETH, and T which accounts 6(23%), 4(15.3%), and 3(11.5%) respectively. The remaining, H4-Ural-2, H1, H3, LAM 5, Manu_ancestor, T3-OSA, Manu2, considered to be less prevalent.

S.No	Binary code	Octal value	SIT	Family	Lineage
1#	■□□■■□□■■■□■■■■■■■□□■■□□■□□■□□■□□□□□□□■	777000377760771	149	T3-ETH	EA-lineage 4
2	■□□■■□□■■■□■■■■■■■□□■■■□■□□■□□□□□□□□□□□■	473577357700001	Orphan	H4-Ural-2	Lineage 4
3	■□□■■■■□■■■■■■■■■■□□■■■□■□□■■■■□□□□□□□□■	703737740001171	3365	CAS1-Delhi	EAI-lineage 3
4	■□□□■■■□■■■□■■■■■■□□■■■□■□□■■■■□□□□□□□□■	774777770000000	Orphan	H1	EA-lineage 4
5	■□□■□■□□■■■■■■□■■■□□■■■□■□□□□□□□□□□□□□■	777000377760771	149	T3-ETH	EA-lineage 4
6	■□□■□■□□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777775720771	121	H3	EA-lineage 4
7	■□□■□■■■■□■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703777774003171	Orphan	CAS1-Delhi	EAI-lineage 3
8	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	776737407760771	Orphan	LAM5	EA-lineage 4
9	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777760771	53	T1	EA-lineage 4
10	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777737777760771	37	T3	EA-lineage 4
11	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777760771	53	T	Lineage 4
12	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777720771	50	H3	EA-lineage 4
13	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777000777760771	Orphan	T3-ETH	EA-lineage 4
14	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777677777741771	Orphan	Manu2	Lineage 4
15	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	473735707700001	Orphan	T3-OSA	EA-lineage 4
16	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777760771	53	T	Lineage 4
17	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777737777760771	37	T3	EA-lineage 4
18	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703777760003471	Orphan	CAS1-Delhi	EAI-lineage 3
19	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777677777777400	Orphan	Manu_ances	Lineage 1 Tur
20	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703377740001771	Orphan	CAS1-Delhi	EAI-lineage 3
21	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703777740003771	26	CAS1-Delhi	EAI-lineage 3
22	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777760000	1793	X2	EA-lineage 4
23	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703777761000200	Orphan	CAS	Lineage 3
24	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	603777761000771	Orphan	CAS1-Delhi	EAI-lineage 3
25	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	777777777760771	53	T	Lineage 4
26	■□□■■■■□■■■■■■□■■■□□■■■□■□□□□■■■■■□□□□■	703777760003471	Orphan o	T3-OSA	EA-lineage 4
27*	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	777776777760731	336	X1	EA
28	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□■□□□□■■■■■■■	777777377720771	183	H3	EA
29	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	777777777420771	777	H3	EA
30	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	53	T1	EA
31	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□	777777760000000	786	34	IO
32	■■■■■■■■■o■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	776777777760771	1129	T1	EA
33	■■■■■■■■■□□□□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777037777760771	New	T1	EA
34	■■■□□□□■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□	703777740000000	1264	34	IO
35	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	53	T1	EA
36	■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	777000377760771	149	T3	EA
37	■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	777000377760771	149	T3	EA
38	■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	777000377760771	149	T3	EA
39	■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■■■□□□□■■■■■■■	777777403760771	1688	LAM9	EA
40	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	777777377720771	183	H3	EA
41	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■	677777777720571	699	H3	EA
42	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	777776777760771	119	X1	EA
43	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■■□■■■	777776777760731	336	X1	EA
44	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777774020771	47	H1	EA
45	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■■□■■■	777776777760731	336	X1	EA
46	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	777777777420731	817	H3	EA
47	■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	777000377760771	149	T3	EA
48	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	53	T1	EA
49	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	53	T1	EA
50	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	776737777760771	New	T1	EA
51	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	777777777760731	52	T1	EA
52	■■■□□□□■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□■■□□■■■	703777740003471	247	CAS	EAI

Spoligotype patterns of 52 shared types and their corresponding lineages/sub lineages of *Mycobacterium tuberculosis* complex strains isolated in two cities / Addis Ababa and Ambo/. CBN: a conformal Bayesian network; EA: Euro American; IO: Indo-Oceanic; EAI: East African-Indian

Spoligotyping patterns of 29 shared types and their corresponding lineages/sub lineages identified from a total of 52 Mycobacterium tuberculosis complex strains isolated in both Ambo city, surroundings and Addis Ababa. Spoligotyping of culture positive isolates were performed, and classified into different families of MTB. Among culture positive isolates from ambo, 12 of them were found under T1 family, 6 were found under T3, 2 isolate under CAS, 4 under x1, 5 isolates under Harlem 1, 1 isolate under Harlem 1, 2 were under T4 and the other 1 isolate were found to be under LAM9 families. There are additional distinctive major Mycobacterium tuberculosis complex strains isolated from Addis Ababa, CAS1-Delhi, T3-ETH, and T isolates consists of 6, 4 and 3 isolates respectively. The rest strains were ranked as H4-Ural-2, H1, H3, LAM 5, Manu_ancestor, T3- OSA, Manu2, one isolate for each lineage. This whole MTB families were categorized under four different lineages, most of MTB culture positive isolates were found under Euro-American lineage according to this study, which is 40 of 52 isolates were Euro-American lineage. The other 2 isolates were found to be Indo-Oceanic, 9 isolates under East-African lineage and one turkey /Lineage1/.

6. Discussion

In the current study, 52 Spoligotyping mycobacterial isolates were found, and 29 distinct patterns, or 55.7% of genetic diversity, were identified. In similar study, heterogenic of spoligotypes strains was confirmed consistent with the 53% of genotype diversity (35). In the current study we have tried to compare the Drug resistance pattern of TBLN from two different cities. This research conclusion shows that genetically diverse strains are present in both study areas., indicates significant migration of people from regions to the cities.

Regarding this investigation 52 isolates were used, of which 48 were susceptible to all first-line anti-TB medications, The remaining three isolates were resistant to both RIF and INH, and one isolate was mono-resistant to INH. Similar with a study, Resistance to isoniazid, ethambutol, and rifampicin rates have been trending significantly downward in Taiwan.(36). In India 65 of the 71 examined isolates were responsive to every first- and second-line anti-TB medication. One sample was mono resistant to rifampicin, while four isolates were resistant to isoniazid. study conducted between /2013-2015/. (37).

The present study found a significant frequency of EPTB in a population with illiteracy, young patients. Comparable studies investigated, that female, lower-level education, middle aged participants was significantly associated with EPTB, which can be attributed to sociocultural factors including illiteracy, short gestation periods, housework, and early marriage. This factor weakens the female immune system and makes them more susceptible to the disease(2) Participants who consume raw milk/meat were observed to be 26(50%), 42(80.7%) respectively. Similarly, 46% of TBLN patients in Africa had a history of consuming raw meat or raw milk. 10–20% of all TBLN have been reported in the past.(38)

In the current study, 52 confirmed M. tuberculosis isolates from two different cities has been verified for drug sensitivity testing and strain identification, and determined to be 7.6% of the isolates were resistant to first-line anti-TB drugs A comparable study in the adjacent nation of Sudan shows that between 2012 and 2016, 10% of M. tuberculosis isolates from TB lymphadenitis patients had first-line treatment resistance DNA sequences.(39). Referring to studies of newly diagnosed cases in Africa, Tanzania had 1.3% MDR-TB cases, Benin had 1.6%, and South Africa had 2.6%. between 2.7% and 3.4% in Mozambique, this is due to the significant rate of treatment

resistance among newly diagnosed cases related to patients' prior use of anti-TB treatment or community exposure to a drug-resistant strain of M. tuberculosis(40).

M. tuberculosis isolates from 225 patients with TB lymphadenitis 15 isolates shown resistance to at least one of the five anti-TB medications, in northern part of Ethiopia in study conducted at 2012 **(40).** The study sample isolates were confirmed for anti-TB drug resistant with GenoType MTBDRplus VER 2.0, and mutations conferring resistant to RIF and INH were detected in 7% of the total isolates. Similar studies conducted, In some parts of Ethiopia, of 91 isolates tested with the same method and 11 % of patients with TB determined to be resistant to RIF and INH between 2016 and 2017(16)**.** In a study carried out in Ethiopia, GenoType MTBDRplus examined 161 chosen isolates for Rifampicin (RMP) and Isoniazid (INH) resistance. 14 isolates (8.7%) were shown to be resistant to at least one of these two first-line anti-tuberculosis drugs by this line probe assay. **(28).**

Correlated to the current study out of 52 culture positive isolates from both cities, four of them were determined to be resistant to INH and three isolates were resistant to both RIF and INH. In the most recent investigation, carried out in Addis Abeba, 151 mycobacterial isolates were examined for their spoligotype patterns and related treatment resistance. Out of 151 isolates tested, 29 were found to be resistance for one of the first-line anti-TB drugs and belong to different lineages between 2020-2021.(41).

In contrast, The Variation of drug resistance rate reported in our study and the earliest studies, Anti-TB drug resistance was more prevalent in some nations than others This could be as a result of patients being exposed to anti-TB medications before visiting medical facilities, variations in the local epidemiology of drug-resistant TB, or differences in the burden of drug resistance among research sites within the nation. This pattern strongly shows that patients are arriving late to receive medical attention. However, the associations between a particular lineage and any drug resistant conferring mutation were considered significant. the prevalent strains identified in this study were SIT53, SIT149, and SIT3336. However, in the SITVIT database These three strains were reported in other studies with lowest prevalence, indicate the predominance of these particular strain for Ambo and Addis Ababa. SIT53 is frequent in central (Ambo)(16).(40).(35). strains SIT53, SIT149, and SIT3336 has been reported in Bahir Dar city moreover, east and west Gojam as well as south and north Gonder (35).

GenoType MTBDRplus, which finds RMP and INH mutations and has a strong correlation with sequencing, was used for genetic characterisation in order to identify resistance mutations. Four of the 52 culture-positive isolates in the current research of MDR-TBLN molecular detection appeared to be resistant to both RIF and INH., which accounts 7.6% of all isolates from both cities. Understanding which genotype families are overrepresented among drug-resistant cases and whether a particular resistant strain is circulating in the general population is important. Therefore, in the current study According to findings from a related study, MDR-TB is connected with several sub lineages in Ethiopia, including CAS1-KILI, CAS1-DELHI, and T3-ETH. A study done in southern Ethiopia revealed a strong correlation between MDR-TB and the Haarlem lineage.(11), considering the fact that our recent investigation found that all Haarlem, T1 and T3 lineages were responsive to RMP and INH. By using PCR-based RD9 typing, all TBLN isolates were identified and confirmed to be Mycobacterium tuberculosis. This detection indicates Mycobacterium tuberculosis strains are responsible for most of TB lymphadenitis cases.

This study's very diversified M. tuberculosis population structure, which included 29 different SITs, showed that the samples were taken from various geographic locations., including orphan or new strain were identified. Among the isolates from Ambo, Lineage- Euro-American / The most common lineages found in this investigation were Lineage 4, accounted for 88.4% and the other two lineages Indo-Oceanic /Lineage 1/ and East-African lineages accounted for 7.6% and 3.8% respectively. Furthermore, isolates from Addis Ababa were identified as similar Lineage with Ambo, major Lineage- Euro-American /Lineage 4/ estimated around 65.3%, followed by East-African Indian /lineage 3/ 30.7% and rare lineage turkey /Lineage 1/ 3.8% of the total isolates.

Related study showed, Lineage 1 /Indo-Oceanic lineage/and East-African lineages were the least prevalent lineages in this study, which indicates relatively low prevalence of these lineages in Ethiopia (30). Additionally, Lineage 1 is reported from Southern Central and North Ethiopia (30). Unlike our finding, a study indicates MDR isolates belonged to the Beijing accounts for most of the MTB resistant strain. Other Strains causing drug-resistant TB cases from other provinces are S, T1, and others.

There are 4 isolates that are resistant to first line anti-TB drugs among TBLN patients in the current study. DST was carried out for all TBLN samples following standard procedures. Furthermore, three isolates were discovered to be resistant to the first-line anti-TB drugs RIF and

INH after testing of all notified M. tuberculosis isolates against isoniazid, rifampicin, and ethambutol, one isolate were mono resistant to INH. The general level of resistance to first line anti-TB drugs in Ethiopia among TB lymphadenitis cases were estimated (39.1%, 48.7%, and 85.7%) respectively (40). Patients with TB lymphadenitis carried drug-resistant TB and MDR-TB, revealing unrecognized rifampicin resistance. It is still difficult to stop the spread of MDR-TB in TB endemic settings because of occurrences of TB lymphadenitis(40).

Additionally, information on the M. tuberculosis population structure worldwide suggests that some TB lineages may have evolved over time to be more likely to spread among particular populations from particular geographic settings and cause disease in a particular group. However, the findings from this study indicates that, T1, T3 and Haarlem strain is endemic in a particular area and common among patients with drug-susceptible TB and resistance TB in Ambo city and CAS1-Delhi, T3-ETH, and T are found to be endemic in Addis Ababa. Further investigation is necessary to comprehend the spread of drug-resistant microorganisms.

7. Limitation

The study had a few minor drawbacks, including a relatively small sample size, the use of mycobacterial isolates from EPTB patients who had visited a particular hospital, which meant that the population of the entire city might not have been represented, and the possibility that regional variations in the prevalence of drug-resistant TB or the drug resistance rate of MTB isolates responsible for extrapulmonary TB might have remained stable throughout the study period.

8. Conclusion

From the finding in this study, we have tried to indicate the association of drug resistant TB with corresponding lineage and molecular epidemiology in patients with tuberculous lymphadenitis. The current study shows an estimated number of drug-resistant mutations in genes and the genetic diversity of drug-resistant M. tuberculosis isolates in patients with TBLN. The most common lineage types in this study were Lineage 3 and Lineage 4, with SIT 125, SIT 149, and SIT 53, each consisting of 4 (27%), 4 (25%) and 3 (18.75%) isolates respectively having substantial clustering frequencies. The predominant lineage from both cities were Euro-American (40/52 76.9%), followed by Indo-Oceanic (2/52; 3.8%), and East-African (9/52;17.30%). Pre-Dominant Sub-lineage from isolates in both cities have been identified. Culture positive isolates were detected to be resistant to RIF and INH and one isolate identified to be Mono-resistant to INH only. our results emphasize the value of ongoing monitoring for MTB resistance TB lymphadenitis at various infection sites.

9. Recommendation

In order to clearly describe the epidemiology of MTBC lineages associated with drug resistance in extrapulmonary tuberculosis patients, a study on a larger number of MTBC isolates is required. Additionally, more molecular epidemiological studies are recommended in lymph node samples in order to clarify their role in TB lymphadenitis. To comprehend the dynamics of drug-resistant TB transmission and boost TBLN control strategies in a specific location, it is advised to employ a better method with the ability to differentiate strains than the Spoligotyping utilized in the current study.

10. REFERENCES

1. Krishnakumariamma K, Ellappan K, Muthuraj M, Tamilarasu K, Kumar SV, Joseph NM. Molecular diagnosis, genetic diversity and drug sensitivity patterns of Mycobacterium tuberculosis strain isolated from tuberculous meningitis patients at a tertiary care hospital in South India. PLoS One. 2020;15(10):e0240257.
2. Abdallah TEM, Mohmmed Toum FE, Bashir OH, Mansoor TI, Yuosif MM, Awad-Elseed Elkhawad M, et al. Epidemiology of extra pulmonary tuberculosis in Eastern Sudan. Asian Pacific Journal of Tropical Biomedicine. 2015;5(6):505-8.
3. Biniam Wondale1, Kwon Keehwan3, Girmay Medhin1, Takele Teklu1,4, Temesgen Mohammed1, Samuel Tolosa1, et al. Molecular epidemiology of clinical Mycobacterium tuberculosis complex isolates in South Omo, Southern Ethiopia. BMC Infectious Diseases. 2020;20:750.
4. V Hollo1 MJvdW. Extrapulmonary tuberculosis in the European Union and European Economic Area, 2002 to 2011. Surveillance and outbreak reports. 2013.
5. MARJORIE P. GOLDEN MD, Yale University School of Medicine and Hospital of Saint Raphael, New Haven, Connecticut. Extrapulmonary Tuberculosis: An Overview. American Family Physician. 2005;72:9.
6. Gashaw F, Zewde A, Tedla E, Wondale B, Mekonnen Y, Erko B, et al. Molecular epidemiology of tuberculosis in northeastern Ethiopia. Research Square. 2021;17:1-10.
7. Singh J, Sankar MM, Kumar P, Couvin D, Rastogi N, Singh S, et al. Genetic diversity and drug susceptibility profile of Mycobacterium tuberculosis isolated from different regions of India. J Infect. 2015;71(2):207-19.
8. Ejo M, Torrea G, Uwizeye C, Kassa M, Girma Y, Bekele T, et al. Genetic diversity of the Mycobacterium tuberculosis complex strains from newly diagnosed tuberculosis patients in Northwest Ethiopia reveals a predominance of East-African-Indian and Euro-American lineages. Int J Infect Dis. 2021;103:72-80.
9. Taye H, Alemu K, Mihret A, Ayalew S, Hailu E, Wood JLN, et al. Epidemiology of Mycobacterium tuberculosis lineages and strain clustering within urban and peri-urban settings in Ethiopia. PLoS One. 2021;16(7):e0253480.
10. Diriba G, Kebede A, Tola HH, Yenew B, Moga S, Addise D, et al. Molecular characterization and drug resistance patterns of Mycobacterium tuberculosis complex in extrapulmonary tuberculosis patients in Addis Ababa, Ethiopia. PLoS One. 2020;15(12):e0243493.
11. Zewdie O, Mihret A, Abebe T, Kebede A, Desta K, Worku A, et al. Genotyping and molecular detection of multidrug-resistant Mycobacterium tuberculosis among tuberculosis lymphadenitis cases in Addis Ababa, Ethiopia. New Microbes New Infect. 2018;21:36-41.
12. Shiferaw Bekele1, 4†, Yohannes Derese1†, Elena Hailu1, Adane Mihret1, Kifle Dagne2, Lawrence Yamuah1 Tropical Diseases, Travel Medicine and Vaccines, et al. . Line-probe assay and molecular typing reveal a potential drug resistant clone of Mycobacterium tuberculosis in Ethiopia Tropical Diseases, Travel Mediciens and Vaccines. 2018;4:15.
13. Houda Ben Ayed1, Makram Koubaa2,3*, Chakib Marrakchi2,3, Khaoula Rekik2,3, Fatma, et al. Extrapulmonary Tuberculosis: Update on the Epidemiology, Risk Factors and Prevention Strategies International Journal of Tropical Diseases. 2018;1.
14. Fantahun Biadglegne1, 3*, Weghata Tesfaye4, Belay Anagaw4, Belay Tessema4,Tewodrose Debebe1, Berhanu Anagaw8, Andargachew Mulu4,6, Ulrich Sack3,5,7, and Arne C. Rodloff2. Tuberculosis Lymphadenitis in Ethiopia. Jpn J Infect Dis. 2013;66:263-8.

15. Syed Beenish Rufai PK, Amit Singh, Suneel Prajapati, Veena Balooni, Sarman Singh. Comparison of Xpert MTB/RIF with Line Probe Assay for Detection of Rifampin-Monoresistant Mycobacterium tuberculosis. Clinical micro- biology. 2014;52:1846–52.
16. Ayalew S, Wegayehu T, Taye H, Wassie L, Girma S, Berg S, et al. Drug Resistance Conferring Mutation and Genetic Diversity of Mycobacterium tuberculosis Isolates in Tuberculosis Lymphadenitis Patients; Ethiopia. Infect Drug Resist. 2021;14:575-84.
17. O. Zewdie1 AM, T. Abebe2, A. Kebede3, K. Desta4, A. Worku5,et.al. Genotyping and molecular detection of multidrug-resistant Mycobacterium tuberculosis among tuberculosis lymphadenitis cases in Addis Ababa, Ethiopia. Elsevier Ltd. 2017;21:36-41.
18. Diriba G, Tola HH, Alemu A, Yenew B, Gamtesa DF, Kebede A. Drug resistance and its risk factors among extrapulmonary tuberculosis in Ethiopia: A systematic review and meta-analysis. PLoS One. 2021;16(10):e0258295.
19. Dan Wamala1, Benon Asiimwe2, Edgar Kigozi2, Gerald Mboowa2, Moses Joloba2 and Gunilla Kallenius3. Clinico-pathological features of tuberculosis due to Mycobacterium tuberculosis Uganda genotype in patients with tuberculous lymphadenitis: a cross sectional study. BMC Biomed central. 2014;14.
20. Getu DiribaID1* HHT, Ayinalem Alemu1, Bazezew Yenew1, Dinka Fikadu Gamtesa1, Abebaw Kebede1,. Drug resistance and its risk factors among extrapulmonary tuberculosis in Ethiopia: A systematic review and meta-analysis. PLOS ONE. 2021;2.
21. Biadglegne F, Tessema B, Rodloff AC, Sack U. Magnitude of gene mutations conferring drug resistance in mycobacterium tuberculosis isolates from lymph node aspirates in ethiopia. Int J Med Sci. 2013;10(11):1589-94.
22. Fantahun Biadglegne1, 3,5, Belay Tessema4, Ulrich Sack3,5 & Arne C. Rodloff2. Drug resistance of Mycobacterium tuberculosis isolates from tuberculosis lymphadenitis patients in Ethiopia. Indian J Med Res. 2014:116-22.
23. Hanif M, Sharma S, Chopra K, Sharma M, Dwivedi K, Sidiq Z, et al. Detection of multidrug resistance and extensively drug resistance among smear-negative extrapulmonary tuberculosis cases in a reference laboratory. Biomedical and Biotechnology Research Journal (BBRJ). 2018;2(2):132.
24. Nicol MP, Wilkinson RJ. The clinical consequences of strain diversity in Mycobacterium tuberculosis. Trans R Soc Trop Med Hyg. 2008;102(10):955-65.
25. Gandhi NR, Brust JC, Moodley P, Weissman D, Heo M, Ning Y, et al. Minimal diversity of drug-resistant Mycobacterium tuberculosis strains, South Africa. Emerg Infect Dis. 2014;20(3):426-33.
26. Korma W, Mihret A, Hussien J, Anthony R, Lakew M, Aseffa A. Clinical, molecular and drug sensitivity pattern of mycobacterial isolates from extra-pulmonary tuberculosis cases in Addis Ababa, Ethiopia. BMC Infect Dis. 2015;15:456.
27. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, et al. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerg Infect Dis. 2013;19(3):460-3.
28. Bekele S, Derese Y, Hailu E, Mihret A, Dagne K, Yamuah L, et al. Line-probe assay and molecular typing reveal a potential drug resistant clone of Mycobacterium tuberculosis in Ethiopia. Trop Dis Travel Med Vaccines. 2018;4:15.
29. Zewdie O, Mihret A, Ameni G, Worku A, Gemechu T, Abebe T. Molecular typing of mycobacteria isolated from tuberculous lymphadenitis cases in Addis Ababa, Ethiopia. Int J Tuberc Lung Dis. 2016;20(11):1529-34.

30. Lobie TA, Woldeamanuel Y, Asrat D, Beyene D, Bjoras M, Aseffa A. Genetic diversity and drug resistance pattern of Mycobacterium tuberculosis strains isolated from pulmonary tuberculosis patients in the Benishangul Gumuz region and its surroundings, Northwest Ethiopia. PLoS One. 2020;15(4):e0231320.
31. Dongsi Lu M, PhD,1,2 Bobbi Heeren, MT(ASCP),2 and W. Michael Dunne, PhD1. Comparison of the Automated Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT) With Löwenstein-Jensen Medium for Recovery of Mycobacteria From Clinical Specimens. American Society for Clinical Pathology. 2013.
32. Pestariati1(CA) MR. Solid Media (Lowenstein Jensen) and Liquid Media (Mycobacteria Growth Indicator Tube) Usage Against Mycobacterium tuberculosis Culture in Sputum Suspect Tuberculosis. Humanistic Network for Science and Technology. 2021;5.
33. Haile B, Tafess K, Zewude A, Yenew B, Siu G, Ameni G. Spoligotyping and drug sensitivity of Mycobacterium tuberculosis isolated from pulmonary tuberculosis patients in the Arsi Zone of southeastern Ethiopia. New Microbes New Infect. 2020;33:100620.
34. Moga S, Bobosha K, Fikadu D, Zerihun B, Diriba G, Amare M, et al. Diagnostic performance of the GenoType MTBDRplus VER 2.0 line probe assay for the detection of isoniazid resistant Mycobacterium tuberculosis in Ethiopia. PLoS One. 2023;18(4):e0284737.
35. Nuru A, Mamo G, Worku A, Admasu A, Medhin G, Pieper R, et al. Genetic Diversity of Mycobacterium tuberculosis Complex Isolated from Tuberculosis Patients in Bahir Dar City and Its Surroundings, Northwest Ethiopia. Biomed Res Int. 2015;2015:174732.
36. Lai CC, Liu WL, Tan CK, Huang YC, Chung KP, Lee MR, et al. Differences in drug resistance profiles of Mycobacterium tuberculosis isolates causing pulmonary and extrapulmonary tuberculosis in a medical centre in Taiwan, 2000-2010. Int J Antimicrob Agents. 2011;38(2):125-9.
37. Sivakumar S, Chandramohan Y, Kathamuthu GR, Sekar G, Kandhasamy D, Padmanaban V, et al. The recent trend in mycobacterial strain diversity among extra pulmonary lymph node tuberculosis and their association with drug resistance and the host immunological response in South India. BMC Infect Dis. 2020;20(1):894.
38. Mekonnen D, Derbie A, Abeje A, Shumet A, Nibret E, Biadglegne F, et al. Epidemiology of tuberculous lymphadenitis in Africa: A systematic review and meta-analysis. PLoS One. 2019;14(4):e0215647.
39. Ibrahim Elhag N, Eltahir EAG. Drug Resistant Mycobacterium tuberculosis Complex Isolates among Patients with Tuberculous Lymphadenitis. Journal of Ancient Diseases & Preventive Remedies. 2016;4(1).
40. Fantahun Biadglegne1, 3,5, Belay Tessema4, Ulrich Sack3,5 & Arne C. Rodloff2. Drug resistance of Mycobacterium tuberculosis isolates from tuberculosis lymphadenitis patients in Ethiopia. Indian J Med Res. 2014;140:116-22.
41. Diriba G, Kebede A, Tola HH, Alemu A, Yenew B, Moga S, et al. Mycobacterial Lineages Associated with Drug Resistance in Patients with Extrapulmonary Tuberculosis in Addis Ababa, Ethiopia. Tuberc Res Treat. 2021;2021:5239529.
42. PROGRAMME NTALC. National Standard Operating Procedures for Tuberculosis Laboratory Diagnosis. NATIONAL TUBERCULOSIS AND LEPROSY CONTROL PROGRAMME. 2017;2.

11. ANNEXES

11.1 SOPS

TITLE

STANDARD OPERATING PROCEDURES ON IDENTIFICATION OF MTBC AND DRUG RESISTANCE TO INH, RIF USING LINE PROBE ASSAY (MTBDR*plus***)**

PURPOSE

This procedure describes the steps required to identify Mycobacterium tuberculosis complex and its resistance to first line anti TB drugs (rifampicin (RIF), isoniazid (INH), drugs using DNA strip technology**.**

SCOPE

This procedure describes the steps required to identify MTBC and its resistance to rifampicin, isoniazid, using DNA strip technology in the TB reference laboratories.

PRINCIPLE

The identification of resistance to rifampicin is enabled by the detection of most significant mutations of the *rpoB* gene (coding for β-sub-unit of the RNA polymerase). For testing of high level isoniazid resistance, the *katG* gene (coding for catalase peroxidase), and for testing of low level isoniazid resistance, the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) is examined. The entire procedure is divided into four steps

1. DNA extraction from EXTR-pulmonary TB / Tuberculosis Lymphadenitis/.

2. A multiplex PCR amplification with biotinylated primers.

3. Hybridization reverse process, which involves

- Chemical denaturation of the amplification products

- Hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes
- Stringent washing
- Addition of a streptavidin/alkaline phosphatase (AP) conjugate
- An AP-mediated staining reaction

4. Evaluation

- Pasting of strips on templates worksheets
- Interpretation of results using the MTBDR*plus* charts

Steps for the identification of MTBC and drug resistance (RIF, INH).

Note: A template ensures the easy and fast interpretation of the banding pattern obtained.

Denaturation	Separation of amplicons in single-stranded DNA
Hybridization	Binding of labelled amplicons to probes
Stringent Wash	Removal of un-specifically bound DNA from probes
Conjugate Reaction	Binding of an enzyme-conjugated protein
Substrate reaction	Enzymatic conversion of dye

SPECIMENS

- FNA Sample collected from Tuberculosis Lymphadenitis patients

EQUIPMENT, SUPPLIES AND REAGENTS

DNA Extraction

EQUIPMENT	**SUPPLIES**	**REAGENTS**
BSC Class II Micro centrifuge Water bath set at 95oC	Filtered pipette tips 100 µL, 1000 µL Screw-capped PCR tubes 1.5 mL, 2.0 mL Fine-tip permanent marker	Molecular biology–grade water (MGW) Distilled water

Pipettes 100–1000 µL Timer Vortex mixer Ultrasonic water bath Thermometer	Paper towels Bench pad/benchkote Biohazard bag Discard container Sterile disposable transfer pipettes Floaters Micro tube racks PPE (disposable gloves, laboratory gowns, N95 respirators, shoe covers/laboratory shoes, bouffant caps) 50.0 mL screw-capped centrifuge tubes Disinfectants:0.5% hypochlorite, 70% alcohol	

Multiplex Amplification

EQUIPMENT	**SUPPLIES**	**REAGENTS**
Thermal cycler Clean cabinet (Dead Air Box) BSC Class II Vortex mixer Pipettes: 0.5–10 µL, 100–1000 µL	Filtered pipette tips: 10 µL, 1000 µL PPE (disposable gloves, laboratory coats) PCR tray and retainer set PCR tubes DNAse and RNAse free 0.2 mL, 1.5 mL and 2.0 mL Fine-tip permanent marker Paper towels Biohazard bag	Primer nucleotide mix (PNM) 10× buffer MgCl2 solution HotStarTaq® polymerase Molecular grade Water /MGW/

	Sharp container Disinfectants: 0.5% hypochlorite, 70% alcohol	

Identification/Hybridization

EQUIPMENT	SUPPLIES	REAGENTS
TwinCubator® Timer Pipettes: 20–200 µL, 100–1000 µL	Discard container Sterile 50.0 mL conical screw cap tubes Sterile 15.0 mL conical screw cap tubes Sterile disposable transfer pipettes Sharpie or permanent marker Tweezers Paper towels Special pen PPE (disposable gloves, laboratory coats) Pipette tips: 200 µL, 1000 µL Disinfectants: 0.5% hypochlorite, 70% alcohol	Membrane strips Hybridization buffer Stringent wash solution. Rinse solution Conjugate concentrate Conjugate buffer Substrate concentrate Substrate buffer Distilled water Molecular grade water (MGW)

DNA Extraction Procedure: Clinical Patient Samples (FNA)

Note: Process FNA using a BSC and appropriate PPE.

1. Process clinical specimens using the NaOH-NALC technique.
2. Label each 2.0 mL centrifuge tube with specimen identification number.
3. In a BSC II use a sterile graduated pipette and transfer 0.5 mL of the decontaminated specimen into the respective tube.
4. Close tube.
5. Centrifuge tube for 15 minutes at 10000rpms using a micro-centrifuge.

6. Discard supernatant.
7. Add 100 µL MGW to each tube.
8. Re-suspend mycobacteria by vortexing gently for 30 seconds.

PCR Amplification

This procedure involves 3 steps:

I. Preparation of master mix: This procedure must be done in a clean, DNA-free room using a clean hood (Dead Air Box)

II. Addition of DNA to master mix

III. Amplification in thermocycler.

I. PREPARATION OF MASTER MIX

1. Remove master mix reagents from the -20^{o}C freezer and allow to thaw out (exception of HotStarTaq®, which should be taken out immediately before use).
2. Place a 2.0 mL micro-centrifuge tube (master mix tube) in a rack and label.
   Add reagents one at a time to the master mix tube according to the quantities listed in the Master Mix Calculation.
   - 35.0 µL PNM mix
   - 5.0 µL PCR buffer
   - 2.0 µL magnesium chloride
   - 2.8 µL MGW
   - 0.2 µL HotStarTaq® polymerase enzyme

   Vortex the master mix tube on low setting.
3. Place 0.2 mL micro-centrifuge tubes plus cover in the PCR tray/base. Take note of the sequence numbers on the tube (e.g., 1,2,3,4…).
   Pipette 45.0 µL of the master mix to each PCR tube.
   Label 0.2 mL micro-centrifuge tubes: one tube per sample plus one for master mix control, one for negative control and one for positive control.
4. Add 5.0 µL MGW to the first tube labelled 1 (master mix control).

5. Close tubes and take to DNA addition room.
6. Place master mix aliquots and DNA under a class II BSC.
7. To each remaining tube of master mix (from tube 2 onwards), add 5 µL of the respective DNA and mix. Add 5 µL positive and negative controls in the last two tubes.

III. AMPLIFICATION IN THERMOCYCLER

- Place 0.2 mL micro-centrifuge tubes with master mix/DNA mixture in the PCR tray inside the thermocycler.
- PCR reactions involve heating at various cycles in order to denature, anneal and elongate the DNA of the specimen.
- The following table illustrates the heating cycles used in the PCR reaction.

STEP	Temperature	Time	Sample Type: Culture	Sample Type: Clinical
1.	95oC	5 minutes	1 cycle	1 cycle
2.	95oC 58oC	30 seconds 2 minutes	10 cycles	10 cycles
3.	95oC 53oC 70oC	25 seconds 40 seconds 40 seconds	20 cycles	30 cycles
4	70oC	8 minutes	1 cycle	1 cycle

At the end of the cycle, remove amplified DNA from the thermocycler and take it to the detection room for hybridization.

- If amplicons cannot be hybridized immediately, they should be stored between +8 and -20oC.

Hybridization

1. Pre warm shaking water bath/TwinCubator® to 45oC.
2. Pre warm solutions HYB and STR to 37–45oC in the water bath before use.
3. Label tray wells with sample number.
4. Dispense 20 µL denaturation solution (blue) in a corner of each of the well to be used.
5. To each respective well, add 20 µL of amplified sample.
6. Mix by pipetting up and down.
7. Incubate at room temperature for 5 minutes.
8. Take out strips using tweezers, and mark sample number with special pen

9. Underneath the colored marker.
10. Carefully add to each well 1.0 mL of pre-warmed hybridization buffer.
11. Place a strip in each respective well in the corner away from the reagents.
12. Gently shake the tray until the solution has a homogenous colour.
13. Place tray in shaking TwinCubator®, and incubate for 30 minutes at 45ºC.
14. Completely aspirate the hybridization buffer.
15. Add 1.0 mL of stringent wash solution to each well.
16. Incubate for 15 minutes at 45oC in the shaking TwinCubator® .
17. Completely remove stringent wash solution (decant and then turn tray upside down on paper towels).
18. Wash each strip once with 1.0 mL rinse solution for 1 minute on shaking TwinCubator® (step 3).
19. Add 900 µL of diluted conjugate to each strip and incubate for 30 minutes on shaking TwinCubator® .
20. Remove solution and wash strip twice for 1 minute with 1.0 mL rinse solution on TwinCubator®
21. Rinse with 1.0 mL distilled water for 1 minute on TwinCubator® .
22. Add 900 µL diluted substrate to each strip and incubate protected from light without shaking.
23. Stop reaction by briefly rinsing twice with 1.0 mL distilled water.
24. Using tweezers, remove strip from tray, dry between papers and tape on to worksheet.
25. Paste strips away from light.
26. Use the chart of reaction zones that accompanies the test kit to interpret results.

SPECIAL PRECAUTIONS

- Always wear appropriate personal protective equipment such as gloves, gowns, N95 respirators etc. when handling live infectious material.
- Carry out sample preparation in a BSC in a BSL2/BSL3.
- Refer to your laboratory safety manual for guidelines on working in a BSL3 laboratory.
- Do not mix reagent from kits with different lot numbers. PNM of MTBDR*plus* should be used when detecting resistance to RIF and INH.
- Store primer/nucleotide mix (PNM) at 2–8ºC upon arrival, isolated from any potential source of contaminating DNA. If longer storage of more than 4 weeks is required, store at –20ºC.
- Store all other kits components at 2–8ºC.
- Do not use reagents above the expiry date.
- Ensure that all reagents and material used for DNA isolation and amplification are free from

DNAases.

- The denaturation solution (DEN) contains less than 2% NaOH and is irritating to the eyes and skin.
- The substrate concentrate (SUB-C) contains dimethyl sulfoxide and is irritating.

QUALITY CONTROL

- A known positive control (extracted fresh from culture such as H37Ra or another that has been previously tested using MGIT 960 DST) is included in each test run.
- MGW is used as negative control.
- If the QC is not correct, refer to the SOP on quality control and what to do if the QC fails, and do corrective action before repeating the test run.
- Document all QC results and any corrective actions performed.

Validate proper performance of test and proper functioning of reagents with the following:

CONTROL ZONE	PURPOSE
Conjugate control (CC) zone	Used to check the binding of the conjugate on the strip and a correct chromogenic reaction. Test is invalid if CC is missing.
Amplification control (AC) zone	The presence of this control zone indicates that the amplification procedure was successful. A missing AC band in the case of a negative result indicates errors during amplification set up or carry over of amplification inhibitors. In case the test is positive for TUB, the respective samples do not have to be repeated.
TUB control zone	This zone hybridizes with amplicons generated from all known members of the MTBC. The presence of this zone indicates that the organism belongs to the MTBC. If this zone missing, the organism does not belong to the MTBC and cannot be evaluated by this test system.
Locus control zones for *rpoB, katG, inhA* (MTBDR*plus*)	Respective locus is present and must always be positive when the TUB has documented the presence of a *M. tuberculosis* strain. If neither the Locus Control probe nor the Wild type or Mutation probes of one of the three genes examined are developed, the test cannot be evaluated.

LIMITATIONS

- This test only works within the limits of the genomic regions that the probes were chosen from.
- The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.

- As with any detection system on hybridization basis, the test system bears the possibility that sequence variations in the genomic region that the primers and probes were chosen from and the detection of which the test was not designed for may lead to false results. Due to the high variability of bacterial genomes it is possible that certain sub-types might not be detected.
- For clinical samples that have not been identified as MTBC, no reaction would occur except the CC and the AC control.
- For clinical samples there may be inhibition and the control locus may not be present.

INTERPRETATION AND REPORTING OF RESULTS

Evaluation and Interpretation of results of the MTBDR*plus* test

- when using the evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet.
- MTBDRplus strip has 27 reaction zones with 6 internal controls: Conjugate control, amplification control, TUB (M. tuberculosis complex) control, rpoB locus control, katG locus control and inhA locus control. Identification of rifampicin resistance is enabled by the detection of mutations of the rpoB gene. Identification of isoniazid resistance and detection of mutations are enabled by the katG gene and the inhA gene.
- rpoB gene locus has 8 wild-type probes and 4 mutation probes.
- katG gene locus has 1 wild-type probe and 2 mutation probes. The gene codes for low level isoniazid resistance.
- inhA gene locus has 2 wild-type probes and 4 mutation probes. The gene codes for high level isoniazid resistance.

TUB gene - the TUB band stains (respective amplicon of MTBC binds to the corresponding probe, which is indicated by the presence of colored line), it is scored as positive (+) in the remark column. The presence of this band indicates that the organism belongs to the MTBC and the **tests (MTBDR*plus*) can be evaluated with this kit.**

Loci control of genes - when the locus control of ***rpoB, katG and inhA*** genes stain, proceed with the evaluation at the loci for MTBDR*plus* kit.

Wild-type probes (*rpoB , katG and inhA)* - When ALL wild-type genes stain (respective amplicon binds to the corresponding wild-type probe, which is indicated by the presence of colored line), it is scored as positive (+). This implies that there is no detectable mutation within the examined region and the strain tested is sensitive to the respective antibiotic at that gene region. Report as positive (+) in the remark column. When one or more wild-type genes do not stain (respective amplicon cannot bind to the corresponding wild-type probe, which is indicated by the absence of colored line), it is scored as negative (-). This implies that there is detectable mutation within the examined region and the strain tested is resistant to the respective antibiotic at that gene region. Report as negative (-) in the remark column.

Mutation probes (*rpoB , katG, inhA)* - When ALL mutation gene do not stain (respective amplicon do not bind to the corresponding mutation probe, which is indicated by the absence of colored line), it is scored as negative (-). This implies that there is no detectable mutation within the examined region and the strain tested is sensitive. Report as negative (-) in the remark column. When one or more of mutation gene stain (respective amplicon binds to the corresponding mutation probe, which is indicated by the presence of colored line), it is scored as positive (+). This implies that there is detectable mutation within the examined region and the strain tested is resistant, Report as positive (+) in the remark column.

Interpretation of results

- Interpret as sensitive if ALL wild type genes stained and ALL mutation genes not stained.
- Interpret as resistant if ONE OR MORE wild type gene(s) is/are not stained (missing).
- Interpret as resistant if ONE OR MORE mutation gene(s) is/are stained (present).

Note: In interpreting results for isoniazid sensitivity/resistance using the MTBDR*plus* kit, the gene loci for the *katG* gene and the *inhA* gene are considered.

a. If both loci are sensitive, report the MTBC sensitive to isoniazid.

b. If one loci has resistant gene (missing of wild type and/or presence of mutation gene) report the MTBC resistant to isoniazid.

Reporting of results

- Report as MTBC identified, sensitive or resistant to isoniazid and/or rifampicin when using MTBDR*plus* kit.

- Report as indeterminate/inconclusive if member of the MTBC but sensitivity pattern to the drugs tested is unclear.
- Report as negative if TUB probe not stained.
- Report resistance with red ink.
- Report sensitive with a blue/black ink.

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.

Weak or no signals except for conjugate control zone

- Quality and/or quantity of extracted DNA do not allow an efficient amplification. Check amplicons on 2% algarose gel. In case no amplicons are visible, repeat DNA extraction and amplification. If necessary, try a different extraction method.
- Incubation temperature was too high.
- The extracted bacterial species cannot be detected by the universal control.

No homogenous staining

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

High background colour

- CON-C and/or SUB-C are too concentrated.
- Washing steps were not performed with necessary care.
- Wash solutions were too cold.

Unexpected result

- Wrong incubation temperature.
- Hybridization buffer and/or stringent wash solution were not properly pre warmed or mixed.
- Contamination of extracted DNA and/or amplification agents with extracted and/or amplified DNA. In case amplification agents are contaminated, a negative control sample also shows respective banding pattern.
- Contamination of neighboring wells by spillage occurred during hybridization process.

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.(42).

12. Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: **Sami Sebri (B.Sc.)**

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: **MR. Melese (MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **MR. Sambel Araya (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.