Genetic Diversity and Drug Resistance Pattern of *Mycobacterium* species among Smear Negative Pulmonary Tuberculosis Patients in Addis Ababa, Ethiopia

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Genetic Diversity and Drug Resistance Pattern of *Mycobacterium* species among Smear Negative Pulmonary Tuberculosis Patients in Addis Ababa, Ethiopia

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A Dissertation Submitted to the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biology (Applied Microbiology)

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ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

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By

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DECLARATION

I, Alem Alemayehu Doyamo (GSR/1458/08), hereby declare that this research dissertation titled "Genetic Diversity and Drug Resistance Pattern of *Mycobacterium* species among Smear Negative Pulmonary Tuberculosis Patients in Addis Ababa, Ethiopia" has been developed by me and has not been submitted to any other institution for award of any academic qualification. The content of the dissertation has not been plagiarized and where works of other researchers have been used, they have been appropriately cited.

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ABSTRACT

Tuberculosis (TB) is being treated empirically in about 38% of patients globally due to difficulty in detecting the *Mycobacterium tuberculosis* complex (MTBC) bacilli with the routine laboratory diagnostic tests, including smear microscopy, GeneXpert and culture. As a result, the situation with smear negative pulmonary tuberculosis (SNPTB) has remained challenging, leading to diagnostic delays and making monitoring treatment outcomes difficult. However, there is scarce information on the actual burden, drug resistance pattern and the strains of MTBC involved in SNPTB. Therefore, this study aimed to evaluate the genetic diversity and drug resistance pattern of MTBC isolates recovered SNPTB patients. A health facility-based case control study was conducted in Addis Ababa, Ethiopia, involving 313 pulmonary TB patients (173 SNPTB and 140 Smear positive PTB (SPPTB)). Laboratory data was generated by collecting sputum specimens from consenting study participant and acid fast staining, GeneXpert and culture were done at Armauer Hansen Research Institute (AHRI) laboratory. Molecular analysis, spoligotyping and whole genome sequencing (WGS) as well as phenotypic drug susceptibility test were done for culture grown MTBC isolates. The revised online MTBC molecular marker database SITVIT2 and appropriate bioinformatics tools were applied to assign lineages and sublineages of MTBC strains. Structured questionnaires were used to assess TB related factors, clinical and imaging findings associated among SNPTB and SPPTB patients. SPSS version 25 and Stata version 17 software were used for data analysis and a P-value < 0.05 was considered statistically significant. Of the 173 SNPTB patients 75 were culture positive. The spoligotyping and WGS analysis of MTBC isolates identified

four major MTBC lineages including lineage 4 (Euro-American), lineage 3 (East-African-Asian), lineage 1 (Indo-Oceanic) and lineage7 (Ethiopian) which had 80.2%, 16.7%, 2.4% and 0.8% proportion, respectively. Lineage 3 MTBC strains were higher among SNPTB patients compared to SPPTB patients while the proportion of lineage 4 MTBC strain was higher among SPPTB than SNPTB (P-value <0.05). A higher proportion of MTBC isolates from SNPTB 16.7% were resistant to one or more first-line anti-TB drugs than SPPTB 12.9% (P-value >0.05). MDR-TB was detected from single SNPTB patients 5.6% (1/18) by both phenotypic and molecular methods whereas none in SPPTB patients (0/33) (P-value >0.05). Although, overall clinical presentations of SNPTB patients resemble those seen in SPPTB patients, prior history of TB was strongly associated with SNPTB (P-value <0.05). There was a significant difference in MTBC strain distribution among SNPTB and among SPPTB patients, which may be responsible for difficulty in MTBC laboratory detection that may need further advanced analysis of sequenced isolates. There was also primary drug resistant TB among culture positive SNPTB patients, which would be otherwise missed by current national protocols. An additional study with larger sample size and more sensitive TB detection approach is recommended for better understanding of SNPTB.

Key Words: GeneXpert, MTBC, NTM, SNPTB, Spoligotyping, WGS

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LIST OF ACKRONIYOUMS/ABBREVIATIONS

AA	Addis Ababa
AAU	Addis Ababa University
AACAHB	Addis Ababa City Administration Health Bureau
AAHB	Addis Ababa Health Bureau
AFB	Acid fast bacilli
AFS	Acid Fast Staining
AHRI	Armauer Hansen Research Institute
AHRIL	Armauer Hansen Research Institute Laboratory
AIDS	Acquired Immunodeficiency Syndrome
ALERT	All Africa Leprosy and TB Control, Rehabilitation and Training Centre
ATS	American Thoracic Society
BAL	Bronchoalveolar Lavage
cAFS	Concentrated Acid-Fast Staining
CNPTB	Culture Negative PTB
CPPTB	Culture Positive PTB
CXR	Chest X-ray
DNA	Deoxyribonucleic Acid
DR	Drug resistance
DOTS	Directly observed treatment, short course
DP	Discriminatory power
EA	Euro-American
EAI	East-African-Indian
DST	Drug susceptibility testing
EMB	Ethambutol
EPTB	Extra pulmonary Tuberculosis
EGCPMTLH	Ethiopian Guidelines for clinical and programmatic management of TB, leprosy and TB/HIV
EPHI	Ethiopian Public Health Institute
HC	Health Centers
HGDI	Hunter-Gaston Discrimination Index

ICSP	International Committee on Systematics of Prokaryotes
HIV	Human Immunodeficiency Virus
IDSA	Infectious Disease Society of America
IO	Indo-Oceanic
INH	Isoniazid
LJ	Lowenstein Jensen
LPA	Line probe assay
LPSN	List of Prokaryotic names with Standing in Nomenclature
MoDR	Mono drug resistant tuberculosis
MDR-TB	Multidrug resistant tuberculosis
MGIT	Mycobacterial growth indicator tube
MIRU	Micro satellite interspersed repetitive unit
MOH	Ministry of health
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MAC	*Mycobacterium avium* complex
NAAT	Nucleic acid amplification techniques
NALC-NaOH	N-acetyl L-cysteine-sodium hydroxide
NTM	Nontuberculous mycobacteria
PCR	Polymerase chain reaction
PTB	Pulmonary TB
PZA	Pyrazinamide
RD	Region of difference
RFLP	Restriction fragment length polymorphisms
RIF	Rifampicin
RNA	Ribonucleic Acid
RR	Rifampicin resistant
RRDR	Rifampicin resistance determining region
RRTB	Rifampicin resistant TB
RTI	Recent transmission index
SIT	Spoligotype international type
SNPTB	Smear negative pulmonary tuberculosis
SPPTB	Smear positive pulmonary tuberculosis

STN	Streptomycin
RNAP	RNA polymerase
RRDR	Rifampicin resistance determining region
SDGs	Sustainable Development Goals
SpolDB	Spoligotyping database
Spoligotyping	Spacer Oligo Nucleotide typing
TB	Tuberculosis
TDR-TB	Totally drug resistant tuberculosis-TB
TOT	Turnaround time
VNTR	Variable Number Tandem Repeat
WGS	Whole genome sequencing
WHO	World Health Organization
XDR- TB	Extensively Drug Resistant
ZN	Ziehl-Neelsen

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1. INTRODUCTION

1.1. Background

Mycobacterium tuberculosis (MTB), as a causative agent of tuberculosis (TB), was identified by the famous scientist R. Koch in 1882 (Koch, 2010) and it is one of the two mycobacterial species of the genus mycobacteria known globally for their mortality and morbidity. The person to person transmission of TB was suggested first by the two famous Greece scientists Isocrates and Aristotle (Barbier & Wirth, 2017) hence then TB has been responsible for more than a million deaths and ten million illness annually worldwide though it is preventable and curable diseases (WHO, 2023b). According to the global TB report, Ethiopia suffers from a TB mortality rate of 17 (10–25) per 100,000 and incidence rate of 126 (85-176) per 100, 0000 population. Smear negative TB or clinically diagnosed TB accounts for m38% of the global TB burden and it has a similar percentage in Ethiopia (MOH, 2024; WHO, 2023b).

Tuberculosis potentially affects any organ of human body from skin to brain, and hence is classified either as pulmonary TB or extra pulmonary TB. Pulmonary TB (PTB) primarily involves the lung and can in some cases exhibit dissemination to other organs, and shares the major global burden by accounting for the 6.2 million of 7.5 million reported cases (WHO, 2023b). PTB is uniquely known for its transmission ability form individual to individual than the extra pulmonary TB which involves any organs other than lung.

PTB can further be classified based on microbiological laboratory diagnosis based on acid fast staining (AFS) as smear negative PTB (SNPTB) or smear positive PTB (SPPTB). This classification has been used to categorize patients by the WHO and national level guidelines for more than a century. Recently classification has been

modified as a result of the widely implemented GeneXpert (Xpert) test, with SNPTB and SPPTB alternatively classified as clinically diagnosed TB versus bacteriologically confirmed TB alternatively according to the Ethiopian national guideline (MoH, 2021).

TB can be drug susceptible, if a patient responds for the anti-TB drug treatment or be drug resistant, if a patient is unable to respond to the anti-TB drug treatment. Drug resistant TB (DR-TB) can be classified as multidrug-resistant TB or extensively drug-resistant TB. Multidrug resistant TB (MDR-TB) refers to resistance to both rifampicin and isoniazid, the two most powerful anti-TB drugs; however, the recent consolidated guidelines by WHO for MDR-TB treatment considers rifampicin resistant TB (RR-TB) as MDR-TB and recommends management as MDR-TB. Extensively drug resistant TB (XDR-TB) is MDR-TB plus resistance to at least one of the two most important classes of drugs in an MDR-TB regimen, resistance to at least one fluoroquinolone (levofloxacin or moxifloxacin) and resistance to at least one other "Group A" drug (bedaquiline or linezolid) (WHO, 2022a).

The WHO's "End TB" Strategy emphasizes early diagnosis of cases and universal drug susceptibility testing (DST) (WHO, 2015). Thus, laboratory confirmation of TB and its drug resistance pattern are essential for correct diagnosis and appropriate and timely treatment for those individuals presenting signs and symptoms of TB. Therefore, formulating a clear and evidence-based policy and strategy as well as the implementation guidelines is essential in every country. However, there is less than 50% coverage of the WHO recommended diagnostic tests worldwide and 38% of PTB patients (WHO, 2023b) are not confirmed bacteriologically while individuals with SNPTB may have comparable disease transmission ability like SPPTB.

Moreover SNPTB patients are at a higher risk of treatment failure and death as a result of loss to follow up than SPPTB patients (Camprubí et al., 2020; MOH, 2024). Thus,effective TB management and control in the whole population will be challenging without addressing SNPTB patients; yet a paucity of information is available on molecular epidemiology and drug resistance of SNPTB globally as well as nationally.

On the other hand, studies show that a noticeable number of SNPTB patients are not really TB cases, and this could be related to the non-specific symptoms of TB and poor specificity of chest X-ray screening. Many medical conditions such as other infections (bacterial, viral, parasitic or fungal) carcinoma, lymphoma, asthma, chronic obstructive lung diseases and other allergic conditions can present as SNPTB cases (Fasih et al., 2915; Serap et al., 2015; Vaghasiya et al., 2016). This results in false positive diagnoses and inappropriate management with TB treatment. Also, technical errors of acid-fast staining can contribute to diagnostic errors. A number of factors may contribute to SNPTB including mycobacterial strain type, host genetics, and technical issues (Figure1).

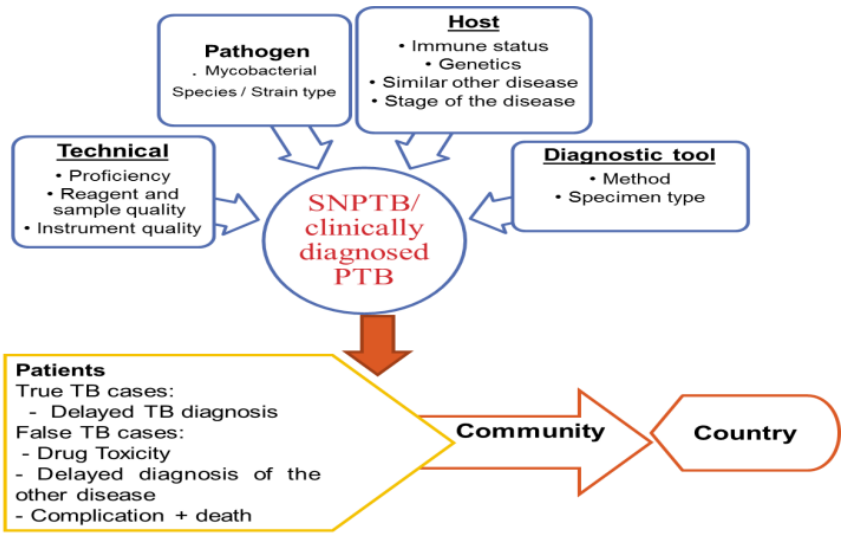


Figure 1. Contributing factors for SNPTB diagnosis and its outcome.

Nontuberculous mycobacteria (NTM) pulmonary infections are among the factors that can be misdiagnosed as SNPTB since it has a similar clinical and laboratory presentation as well as a higher smear negativity rate. Although, NTM pulmonary infection needs accurate clinical and laboratory diagnosis as well as different drug treatment from MTBC infection as indicated in the American Thoracic Society (AST) or the Infectious Diseases Society of America (ATS/IDSA) diagnostic guideline for NTM infection (Griffith et al., 2007). However, during a routine culture isolation of MTBC, most Ethiopian mycobacterial laboratories have been reporting NTM, more than 7% prevalence among PTB patients (Alemayehu et al., 2022; Kahase et al., 2021), without further characterization to species level which is important for determining their clinical relevance and NTM disease management.

Therefore, all these gaps demand a continuous research work for evidence-based TB control policy and strategy. However, MTBC molecular characterization and drug resistance (DR) studies conducted in Ethiopia have not been addressed SNPTB patients as well as NTM unlike SPPTB patients. Therefore, the current study aimed to precisely characterize the mycobacterial isolates and their DR pattern among SNPTB patients.

1.2. Genus *Mycobacterium*

It has been postulated that the genus *Mycobacterium* is originated before 150 million years ago (Hayman, 1984) and it is in the family Mycobacteriacea, Order Mycobacteriales, Phylum Actinobacteria. According the List of Prokaryotic names with Standing in Nomenclature (LPSN) database, the genus has 280 registered species until July 9 2024. Out of these, 211 are validly published under the International Committee on Systematics of Prokaryotes (ICSP), 197 with correct name, and the

remaining 14 with synonyms (Parte et al., 2020). The genus consists of predominantly free-living environmental species; however, a subset of them can induce infections of diverse severity in both human and animal hosts. These include the NTM groups, the *Mycobacterium tuberculosis* complex group (MTBC) and *M. leprea*, the causative bacilli of leprosy. The phylogeny of the genus *Mycobacterium* with comprehensive grouping of its species is shown in Figure 2.

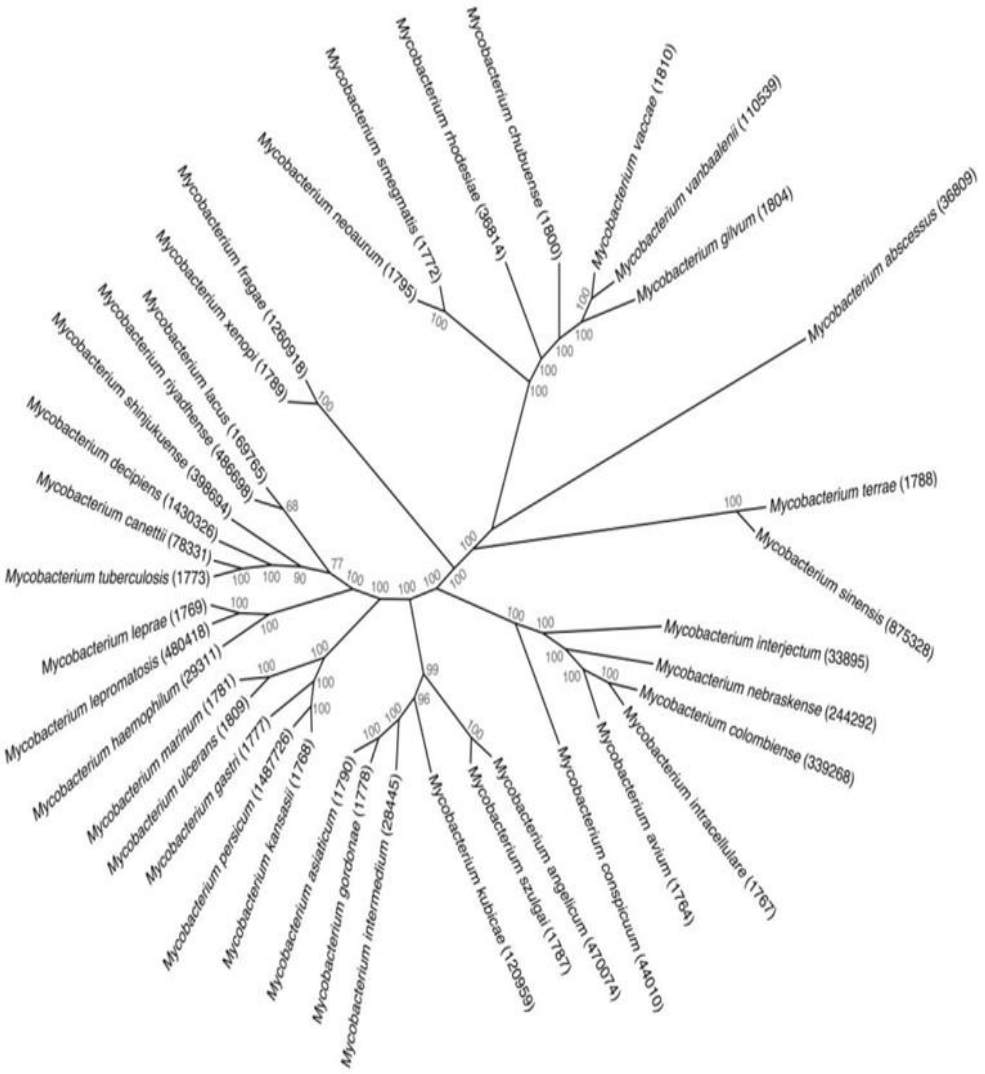


Figure 2. The phylogenetic tree of the genus *Mycobacterium* (Orgeur et al., 2024). Taxonomy IDs of selected *Mycobacterium* spp. are indicated in brackets

1.2.1. Nontuberculous mycobacteria (NTM)

NTM are environmental species of the genus *Mycobacterium* (Gupta et al., 2018) and can be classified as a slow-growers including the *M. avium Complex* (MAC) and *M. simiae,* that require more than seven days to form colonies like MTBC and rapid grower such as *M. fortuitum* and *M. abscesses* complex which require less than seven days to form colonies (Schiff et al., 2019). The majority of the NTM are recovered from environmental sources including water and soil. Unlike other mycobacterial infections, human to human transmission of NTM has not been widely reported and hence human infection is thought to be acquired from environmental exposures (Covert et al., 1999). However, whole genome sequencing (WGS) and epidemiological studies have reported transmission of NTM among cystic fibrosis patients, particularly *M. abscess* complex and MAC species (Bryant et al., 2013; Hasan et al., 2021).

Moreover NTMs are becoming epidemiologically important since their clinical significance is increasingly recognized (Lin et al., 2018; Ratnatunga et al., 2020). In Western countries, MAC (Gopalaswamy et al., 2020; Lee et al., 2021) and in parts of Africa, *M. kansasii* and *M. abscessus* complex (Okoi et al., 2017) have been the most common NTM species that are responsible for pulmonary infections. However, unlike in the developed countries, the distribution of NTM and their disease prevalence are not fully understood in the developing countries, including Ethiopia.

1.2.2. *Mycobacterium tuberculosis* complex (MTBC) and its genome

MTBC, usually called ‘tubercle bacilli’, are a group of closely related bacteria responsible for tuberculosis. This complex has different strains which are further stratified into distinct lineages based on host adaptation i.e., human adapted lineages

and animal adapted lineages. These lineages are evolved through clonal expansion from a common ancestor of *M. canettii*-like tubercle bacilli as descendant clade through a recombinogenic process. Currently, the MTBC consists of eleven species including the human adapted *M. tuberculosis* (lineage 1, 2, 3, 4, 7 and 8) and *M. africanum* (lineage 5, 6, 9 and 10) and animal adapted *M. bovis* and the Bacillus Calmette–GuYPER (BCG) strain, *M. microti, M. pinnipedii, M. caprae*, and *M. canettii, M. orygis, M. suricattae and M. mungi* (Guyeux et al., 2024).

It has been shown that the genome of MTBC has been evolving even in a controlled environment (Ioerger Thomas et al., 2010). Recently, Chitale et al (2022) reported the genome size of *M. tuberculosis* H37Rv as 4417942 bp, which is slightly higher than the genome size of the *M. tuberculosis* H37Rv reference strain sequenced initially in 1998 (Cole et al., 1998). The newly sequenced genome has additional 6.4kb of sequence consisting mainly repetitive Deoxyribonucleic acid (DNA) encoding an insertions in PE/PPE genes, IS6110 transposes, and new paralogs of esxN and esxJ genes (Chitale et al., 2022) that were apparently omitted in the original genome assembly (Cole et al., 1998). The genome is circular, comprising 4.4 million base pairs and about 4,000 genes (Figure 3).

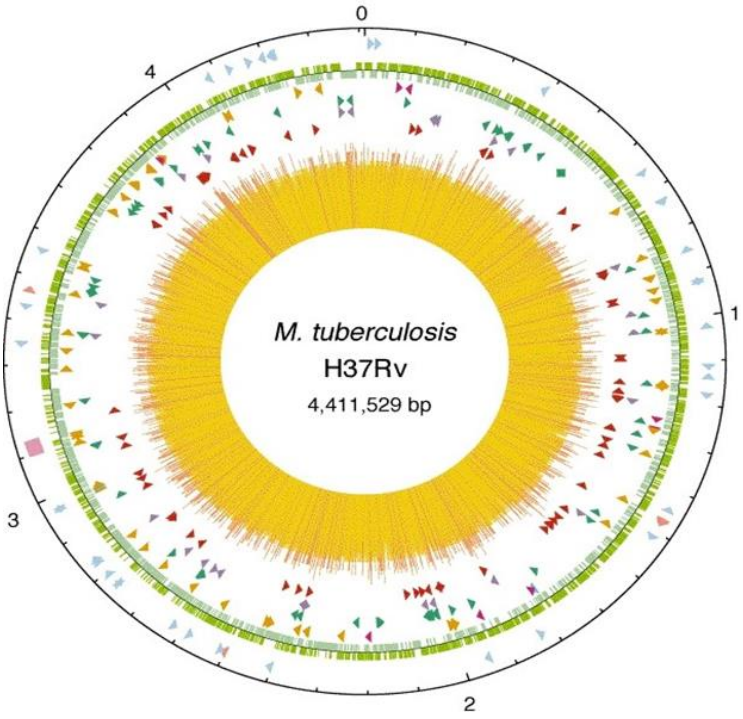


Figure 3. A circular genome of *M. tuberculosis* H37Rv, an illustrative presentation adopted from Cole et al. (1998).
The scale in *M. tuberculosis genome* is displayed in the outer circle, where 0 denotes the replication origin. The external ring designates the locations of the direct repeat region (pink cube) and stable RNA genes (tRNA are blue, others are pink). The second dark green ring shows the coding sequence by strand (clockwise and anticlockwise, the light green ring). Repetitive DNA (insertion sequences, orange) are represented in the third ring. The 13E12RFP family (dark pink, prophage blue) is represented in the fourth ring, the PPE family members' positions (green), PF family members' positions (purple, excluding PGRS) in the fifth ring, and the positions of the PGRS sequences (dark red) shown in the last ring. The G+C content is shown by the histogram (middle), where < 65% G+C is denoted by yellow bars and > 65% G+C is denoted by red bars.

The genome of MTBC is characterized by a high G-C content (65.6%), and 52% of its genome encodes functional proteins (Camus et al., 2002; Cole et al., 1998). Although most of the genes encode enzymes involved in lipid metabolism (Cole et al. 1998), there are also the necessary genes for the catabolic TCA cycle, the anabolic pentose phosphate pathway, and the glycosylate cycle, which converts lipids into carbon. These all contribute to bacilli adaptation in a range of environments, including the oxygen-rich lung, the center of a caseous granuloma, and within macrophages (Swanepoel & Loots, 2014).

Moreover, the Insertion Sequences (IS), polymorphic G-C rich repetitive sequences (PGRS), direct repeat (DR) regions and major polymorphic tandem repeats (MPTR) are among the types of repetitive DNA sequences that are found in the genome (Poulet and Cole, 1995) which enable the development of molecular genotyping methods.

Despite variations in virulence determinants, host tropism and phenotypic attributes members of the MTBC strain share identical 16S rRNA sequences at the nucleotide level and have 99.9% similarity in DNA that contribute for sequence homogeneity (Brosch et al., 2002) which is consistent even between different species of MTBC like *M bovis* and *M. tuberculosis* (Garnier et al., 2003).

1.3. Phylogeny and global distribution of MTBC

In the genus Mycobacterium, including *M. avium* and the MTBC, a number of transitions has been clarified. According to the analysis of deletions and sequence polymorphisms in five selected genes, *katG, gyrA, oxyR, pncA, mmpl6* and *TbD1*, the evolutionary pathway of tubercle bacilli has been proposed (Barbier & Wirth, 2016). The ancient MTBC lineages 1, 5, 6 and 10 have deletion in 1 region (TbD1) whereas, TbD1 is absent in lineages 2, 3, and 4 that are considered as evolutionary modern lineages. Lineage 7 is considered an intermediate between ancient and modern lineages (Barbier & Wirth, 2016; R. Brosch et al., 2002) (Figure 4) (Galagan, 2014).

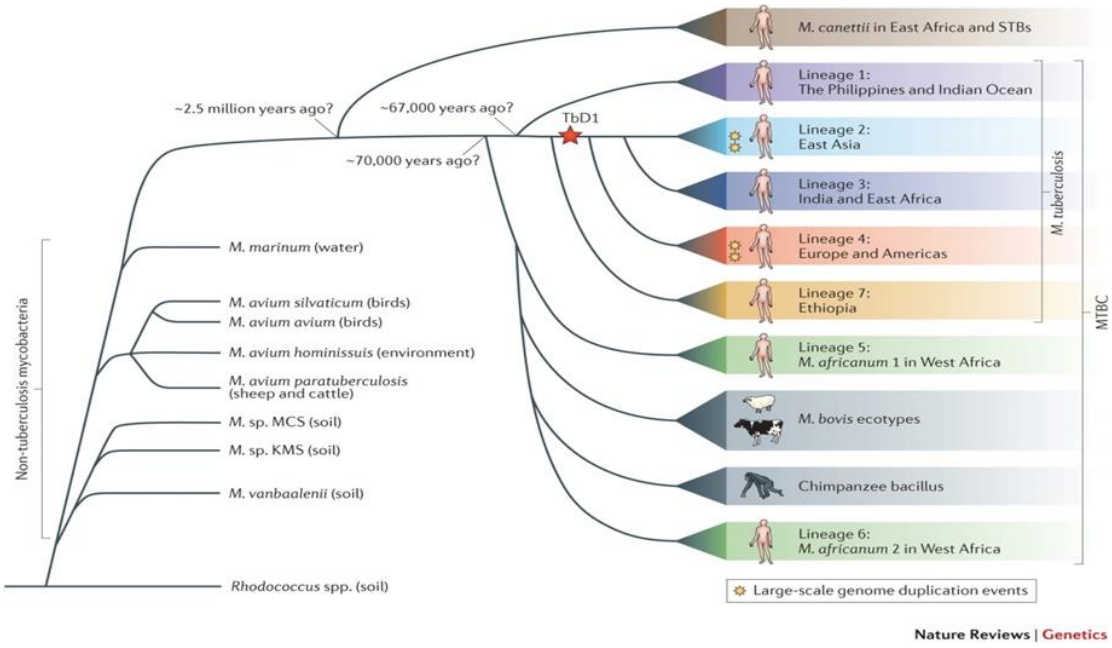


Figure 4. Evolutionary link between the MTBC and certain species of mycobacteria (Galagan, 2014).

The dates of branching events are only rough calculation. TbD1 designates the deletion event specific for modern lineages 2, 3 and 4. Evolutionary distances are not to scale. STBs: smooth tubercle bacilli.

The advancement of molecular methods including genome sequencing has enabled the delineation of MTBC species and clarification of strain evolution. For instance, the animal-adapted *M. bovis* branched from human-adapted lineage of *M. africanum* that is currently restricted to West Africa. Moreover, MTBC species exhibit a remarkable similarity that suggests a common ancestor *M. canettii*. For instance, differences in the genome size(Gutierrez et al., 2005) and colony morphology (Jia et al., 2017). As well as gene transfer mechanism (Supply et al., 2013) are detected between *M. canettii* and MTBC, a descendant lineages.

The evolutionary lineages of MTBC has been extended to ten lineages (Figure 5) and 85 sub lineages based on single nucleotide polymorphism (SNP) analysis (Guyeux et al., 2024; Orgeur et al., 2024). These includes the six lineages of *M. tuberculosis* sensu stricto i.e., lineage 1 (Indo-Oceanic), lineage 2 (East-Asian), lineage 3 (East-

African-Indian), lineage 4 (Euro-American), lineage 7 (Ethiopian) which also is predicted as *M. africanum* based on spoligotyping method, lineage 8 initially discovered in Rwanda and Uganda (Napier et al., 2020), *M. tuberculosis var.* africanum involving lineage 5 and 6, found mostly in West Africa, but also sporadically in eastern Africa, lineage 9 (Somalia and Djibouti), and the newly identified lineage 10 that represents a sister clade to lineage 6 found in central Africa and characterized by a new region of deletion, IS6110 insertions, and 243 SNPs (Guyeux et al., 2024; M. L. Silva et al., 2022).

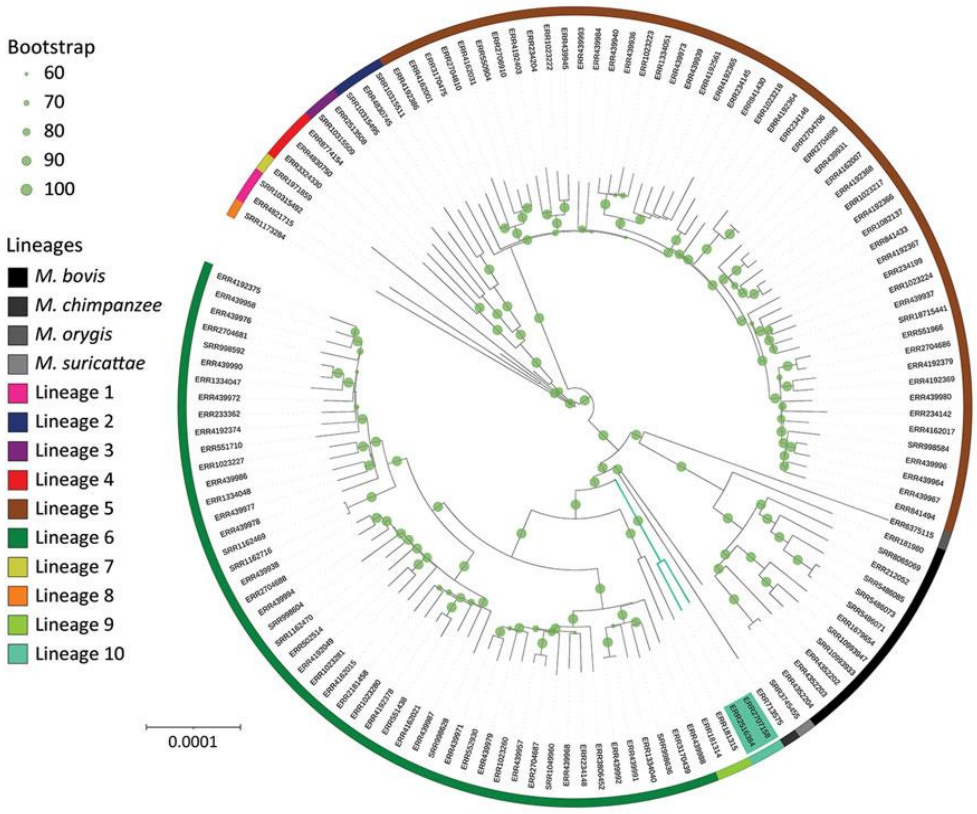


Figure 5. Global MTBC phylogeny including the newly identified lineage10 strains (green) (Guyeux et al., 2024).

Phylogeny was rooted with *M. canettii*, subsequently removed for better visualization. Bootstrap support was computed using 100 replicates and shown when ≥0.6. Circles indicate the large support of almost all branches, especially of lineage10 and its sister branches. Scale bar indicates nucleotide substitutions per site.

1.3.1. **Global distribution of MTBC lineages**

As shown in figure 6, the map of MTBC geographic distribution, MTBC strains have a distinct phylogeographic population structure. For instance, lineage 4 (Euro-American) is the most prevalent MTBC lineage, believed to have originated in Europe. The lineage's expansion to the other parts of the world may be as a result of intercontinental trade and expansionism (Gagneux, 2018; Napier et al., 2020). This lineage is notably associated with pulmonary TB, particularly in regions with high HIV and TB co-infection rates (Wiens et al., 2018). Lineage 3 that originated in south Asia, represents the most common lineage in Africa together with lineage 4, and is prominently associated with drug resistance. Clades 3.5 and 3.1 are strongly associated with eastern Africa, including Sudan and Ethiopia (Shuaib et al., 2022). Beside lineages 3 and 4, Lineage 7, though less common in Ethiopia is unique to the North and Western parts of the country (Getahun et al., 2024).

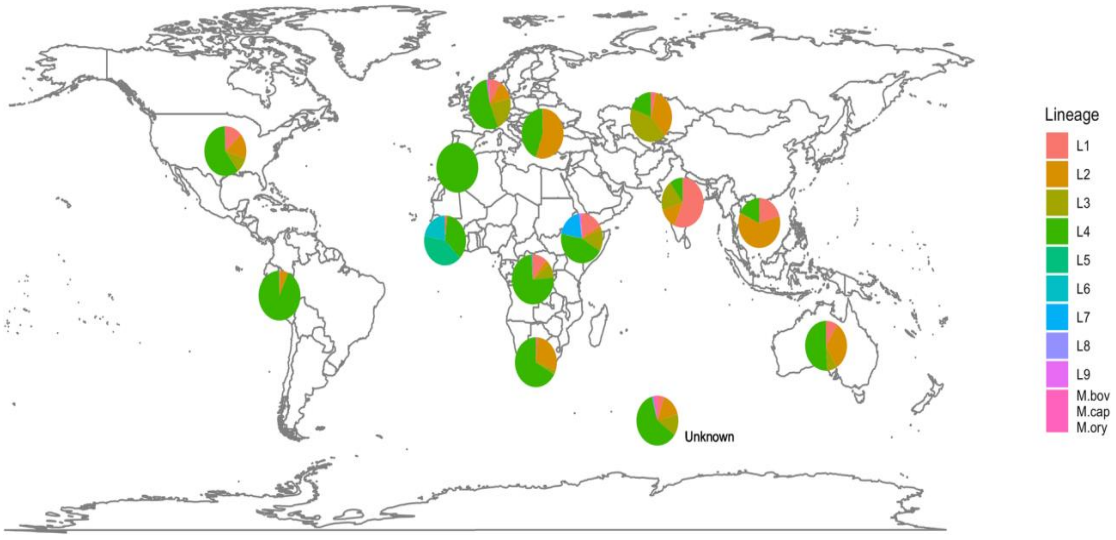


Figure 6. The global distribution of MTBC isolates (Napier et al., 2020)

The most common spoligotype families populated globally as well as in the continent are T, Haarlem (H), Latin-American-Mediterranean (LAM), EAI, MANU, CAS,

Beijing, Cameroon, X, S, Ghana, URAL, TUR, Uganda and H37Rv, AFRI, and West African (Chihota et al., 2018; Salvato et al., 2019). The corresponding WGS sublineages of some spoligotype families are shown in figure 7. MTBC sub-lineages vary based on IS6110 copy number, such as the variation in the EAI lineage (lineage3) related to absence and presence of spacers. Absence of distinct spacers are the specific spoligotype hallmark of the CAS, CAS1-Delhi (also referred to as spoligotype international type 26 [SIT26]) and CAS1-Kilimanjaro (SIT21) family, that is prevalent in East Africa including Ethiopia (Brudey et al., 2006).

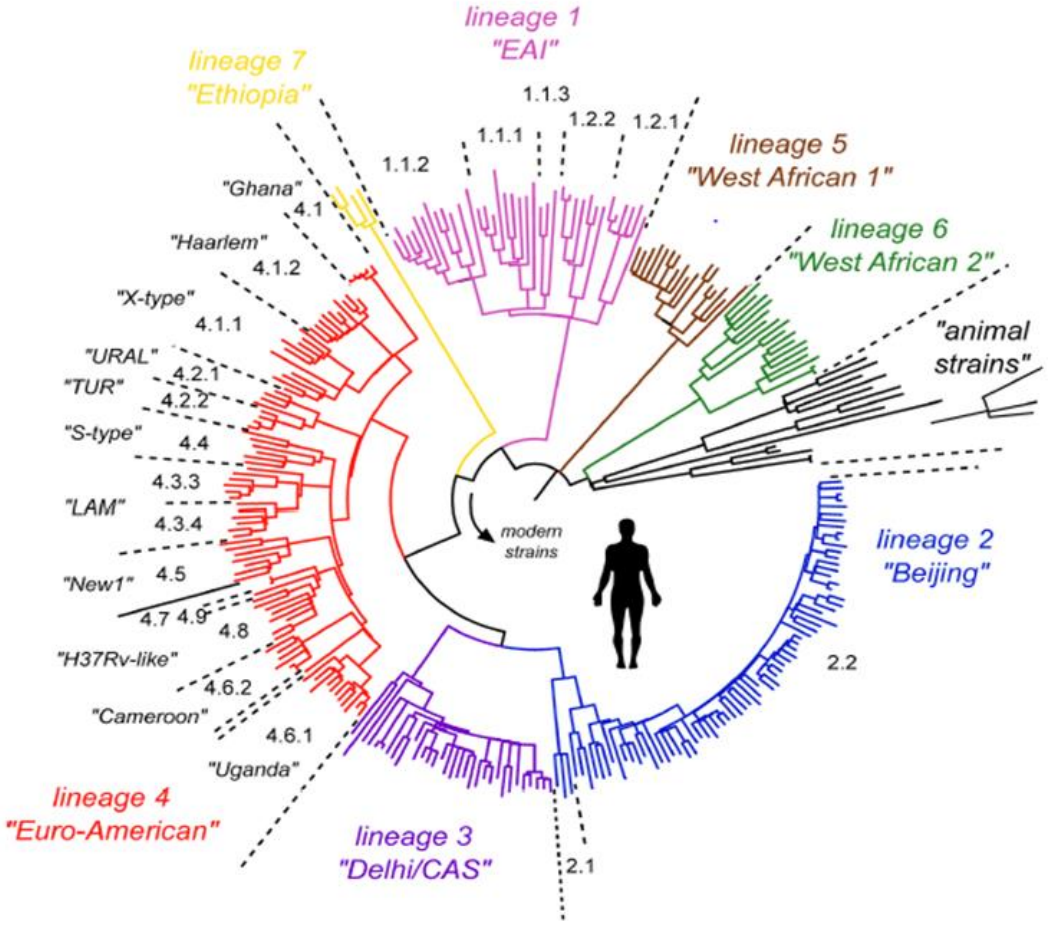


Figure 7. A neighbour-joining tree displaying the phylogenetic structure of the MTBC lineages and sub-lineages (Niemann et al. 2016).

1.3.2. Overview of MTBC lineages in Ethiopia

In Africa several previously unknown lineages of MTBC have been identified during the past decade including MTBC lineage 7 (Ethiopia) in the Horn of Africa and lineage 8 in the African Great Lakes region (Coscolla et al., 2021; Jean Claude Semuto Ngabonziza et al., 2020). *M. africanum* lineage 9 was found only in Djibouti and Somalia (Folliero et al., 2024), and *M. africanum* lineage 10 in Central Africa (Guyeux et al., 2024). Beside the globally populated lineage 4 and 3, the well-known *M. africanum* affiliated lineages, lineage 5 and lineage 6 contribute significantly to TB burden, typically in West Africa (Guyeux et al., 2024).

According to a recent population genetics study in Ethiopia from spoligotyping and MIRU-VNTR data base analysis lineage 4 (70.6%) followed by lineage 3 (25.9%), lineage 1 (1.8%), and lineage 7 (1.6%) comprised the majority of lineages identified. However, the proportion of lineages 4 and 3 varied from region to region (Getahun et al., 2024). In different spoligotyping based studies including the study by Getahun et.al. al. SIT149, SIT53, SIT25, SIT37 and SIT26 are the five most dominant patterns in the country. Moreover, lineage 3, SIT21 and lineage 4, SIT149 and SIT53 were reported as the SITs frequently associated with drug resistance (Getahun et al., 2024).

MTBC lineages are well studied either in the all TB patients or separately among SPPTB which exhibits a similar lineage distribution with the general PTB patients in Ethiopia including Addis Ababa i.e., MTBC lineage 4 is a predominant strain, followed by lineage 3, lineage 7 and/or lineage 11 (Bedewi et al., 2017; Damena et al., 2019; Mekonnen et al., 2023; Merid et al., 2021; Tilahun et al., 2018; Wondale et al., 2020; Worku et al., 2022). However, there is no published data on MTBC lineage distribution mong SNPTB in the study area or in the country as well as globally

except some experimental analysis of AFB test positivity vs. MTBC lineage type (Osei-Wusu et al., 2021). However, understanding MTBC strain diversity especially in SNPTB is crucial for better recognition of TB disease pathogenesis or clinical outcome (Du et al., 2023) , improve diagnostics and therapeutics since TB disease out come and response to ant TB treatment affected by MTBC strain type(Ordaz-Vázquez et al., 2023). Therefore, the current research sought MTBC strain diversity among SNPYB patients.

1.4.Immunology of Tuberculosis

M. tuberculosis have a well-known virulence factors such as ESAT-6/CFP-10, heat shock proteins, cord factor, mycolic acid etc., which enable the MTB bacilli to resist desiccation and chemical damage, form granulomatous lesions, suppresses macrophage function as well as evade and modulate the host responses (Rahlwes et al., 2023; Ramon-Luing et al., 2023). The complex interaction between MTB and the host immune system contribute for TB immunopathogenesis (Carabalí-Isajar et al., 2023; Sankar & Mishra, 2023; Wolf et al., 2008).

The pathogenesis of tuberculosis starts from acquiring the TB bacilli primarily via respiratory droplets from individuals with active pulmonary TB during coughs, sneezes, or speaks, that generates aerosols containing MTB bacilli that can be inhaled via the respiratory route. This results in different out comes in a newly infected individual whom ~50%-80% will completely clear the initial infection and the remaining ~20%-50% will develop active TB (~5%) or latent TB (~95%) of which ~5%-10% will have a TB reactivation later in life (Dheda et al., 2010).

If individuals are diagnosed with TB within 1.5-2 years of newly acquired infection it is called primary active TB. If not the other form of TB, latent TB will occurs in

newly infected individuals whose TB does not develop during within 1.5- 2 years. Latent TB can lead to one of three outcomes: staying infected indefinitely and never getting sick; gradually recovering from their infection; or developing active TB disease later which is commonly referred to as reactivation(Carabalí-Isajar et al., 2023) that frequently caused by factors like HIV infection, malnutrition, or immunosuppressive therapy (D. R. Silva et al., 2018).

Primary Immune Response to Tuberculosis

The inhaled droplets carrying the bacteria reach the alveoli in the lungs which is the initial stage of MTB infection that could result in ‘primary infection’ which alveolar macrophages engulf the bacilli, however, the bacteria will survive in the macrophage mainly by inhibition of phagosome-lysosome fusion and replicate within the macrophages. Then, the innate immune response primarily neutrophils and dendritic cells are recruited with some infected macrophages migrate to regional lymph nodes, activating the adaptive immune system followed by ‘granuloma’ formation.

Innate Immune Response to Tuberculosis

Limiting early bacterial multiplication is a critical function of the innate immune system, which is the first line of protection against MTB. Alveolar macrophages in the lungs are the main cells engulfing MTB bacilli. However, MTB survives internalization within the phagosome by blocking the phagosome's fusion with the lysosome by arresting phagosome maturation and acidification by using its products including lipoarabinomannan, trehalose dimycolate, or acyltrehalose-containing glycolipids and other additional mechanisms (Pethe et al., 2004; Queval et al., 2017). The other component of innate immune response, dendritic cells play a central role in the early immune response by capturing and presenting MTB antigens to T cells.

They activate the adaptive immune response and are essential for the development of protective immunity (H. Kim & Shin, 2022; Mihret, 2012). Moreover, by releasing reactive oxygen species (ROS), antimicrobial peptides and additional effector mechanisms neutrophils try to eradicate MTB as the innate immune response mechanism (Gaffney et al., 2022; E. Warren et al., 2017). However, it is reported that neutrophils can facilitate local dissemination of granulomas and thereby enable the spread of infection beside their influence in bacillary load during both innate and adaptive immune responses (Gaffney et al., 2022; Hult et al., 2021).

In addition to the cellular components of innate immune response, TNF-α, IL-1β, IL-6, and IL-12 are examples of cytokines that are essential for immune response coordination. TNF-α has a crucial role in the development of granulomas and macrophage activation (Boni et al., 2022).

Adaptive Immune Response to Tuberculosis

Following the innate immune response, the adaptive immune system is triggered and is essential for managing the infection. T lymphocytes especially CD4+ T cells, CD8+ T cells, and granuloma development are the main elements of the adaptive immune response to tuberculosis.

In the lymph nodes, dendritic cells expose CD4+ T cells to MTB antigens. The adaptive immune response is characterized by the activation of Th1 cells, which generate interferon-gamma (IFN-γ). The activation of macrophages, which increases their capacity to eliminate MTB, depends on IFN-γ (de Martino et al., 2019). By using MHC Class I molecules, CD8+ cytotoxic T lymphocytes (CTLs) are able to identify infected cells that carry MTB antigens. These T cells destroy infected cells directly by

producing granzymes and perforins, which cause the infected host cells to undergo apoptosis (Grotzke & Lewinsohn, 2005; Wolf et al., 2008).

Granuloma development is one of the most important aspects of the immunological response to tuberculosis. Granulomas are collections of immune cells, including dendritic cells, T cells, and macrophages that stop the infection from spreading (Hult et al., 2021; H. Kim & Shin, 2022) which has been also considered as host 'disease tolerance' mechanism (Divangahi, 2018). Although MTB can persist in granulomas in a dormant condition and cause latent tuberculosis infection, granulomas restrict bacterial multiplication. Granulomas may sometimes degrade, which could result in the infection spreading and the emergence of active TB (Elkington et al., 2022; Gideon & Flynn, 2011).

Secondary Immune Response to Tuberculosis

When the immune system comes into contact with MTB again, either as a result of re-exposure or the reactivation of a latent infection (D. R. Silva et al., 2018), the secondary immunological response takes place. Because memory T cells and memory B cells from the original infection are present, the secondary immune response is quicker and more effective. Memory Th1 cells and memory CD8+ T cells both rapidly identify MTB antigens upon re-exposure, generating IFN-γ and eliminating infected cells directly, respectively. Granulomas may reawaken in latent TB patients when the immune system is compromised, which can result in MTB proliferation and the emergence of active TB disease.

In summary, both innate and adaptive immunity are involved in the complex immune response to tuberculosis. Although macrophages, neutrophils, and cytokines are the first defense components used by the innate immune system to try to manage MTB,

the adaptive immune response in particular, T lymphocytes is ultimately responsible for managing the infection. Although granuloma formation aids in bacterial containment, MTB's capacity to live inside macrophages and develop latent infections is still a problem. TB is a chronic and occasionally reactivating disease because of the immune evasion strategies used by MTB, which also contribute to the chronic infection.

Tuberculosis Immunopathogenesis among SNPTB and SPPTB patients

The clinical outcome of TB also affected by the host immune response (Carabalí-Isajar et al., 2023) and studies has been shown that the immunopathogenesis of tuberculosis is different among clinically diagnosed individuals, SNPTB and those who have bacteriological confirmation individuals, SPPTB. Although both groups show immune responses to contain the infection, patients with bacteriological confirmation frequently have more severe illness because of larger bacterial burdens and a complex immune evasion used by MTB such as granuloma manipulation, immune response regulation (e.g., via IL-10) (Muchsin et al., 2022; O'Leary et al., 2011) and prevention of phagosome-lysosome fusion, enabling on going survival and multiplication than SNPTB who have a higher levels of pro-inflammatory cytokines (like TNF-α and IL-1β) (X. Li et al., 2024).

It has been showed that change in lymphocyte subsets in SNPTB is different from that of SPPTB is also, for instance a higher proportion of B lymphocytes are detected in SNPTB unlike SPPTB whom dominated by CD4+ T lymphocytes (Yang & Feng, 2023). As a result the immunopthogenesis difference among SNPTB and SPPTB has been explored by researchers to identify a potential immunological biomarkers for TB

diagnostic and monitoring ant-TB drug treatment response in these group patients (Carabalí-Isajar et al., 2023; Esmael et al., 2022).

1.5. Diagnosis of tuberculosis

The WHO recommended comprehensive diagnosis of PTB which involves patient's medical history, physical examination, chest imaging, TB blood or skin tests if indicated, bacteriologic examination that includes sputum smear microscopy, nucleic acid amplification testing, culture and drug susceptibility testing. However, each country's TB diagnosis guidelines can be customized based on the country's disease burden and socioeconomic level. Though smear microscopy has been utilized in areas without functional Xpert machines beyond its use for monitoring primarily drug susceptible TB, the updated national guide line recommends Xpert as the initial diagnostic test (MoH, 2021). For PTB patients with smear microscopy or Xpert test negative results, clinicians commonly use clinical assessment with imaging findings to rule in or out PTB in Ethiopia (MoH, 2021).

1.5.1. Clinical diagnosis of pulmonary TB

The symptoms of PTB which affects the lower respiratory system yet can disseminate and cause infection in other organs (named milliary TB) includes fever, sweats, cough, blood stained sputum (Daniel, 2006; Pease, 1940), loss of appetite and unintentional weight loss, chills, fatigue and malaise (WHO, 2024). In Ethiopia, individuals with at least a two week history of cough, fever, night sweats, weight loss or chest X-ray (CXR) infiltrates is suggestive of PTB and considered for further laboratory assessment (MoH, 2021).

1.5.2. Chest imaging findings

Consolidation and pulmonary cavitation was first identified as pathologic signs of PTB in 1819 by the French Theophile Laennac (Barberis et al., 2017). TB screening as well as diagnosis for microbiologically negative TB cases has been supported by imaging technologies including chest X-ray (CXR) and Ultrasonography (Cozzi et al., 2024; WHO, 2024), though these findings are not specific for PTB. PTB can be classified as cavitary or non-cavitary based on imaging findings which has implications on the disease severity and transmission potential. Though unable to detect DR-TB and monitor patients anti-TB drug treatment outcome, chest imaging findings has been the main radiological imaging tool for smear or Xpert negative PTB supporting compatible clinical sign and symptom evaluation in Ethiopia (MoH, 2021).

1.5.3. Laboratory diagnosis of tuberculosis

Laboratory confirmation of TB was realized after R. Koch isolated and cultivated the bacteria and further inoculated it into laboratory animals. Moreover the detection of the bacillus for the first time was made possible by applying the methylene blue staining recommended by Paul Ehrlich in 1882 (Barberis et al., 2017). Sputum smear microscopy, Xpert MTB/RIF, Light Emitting Diode LED-Fluorescence Microscopy (LED- FM), BACTEC *Mycobacterium* Growth Indicator Tube (MGIT) 960 system, lateral flow urine lipoarabinomannan assay (LF-LAM), loop-mediated isothermal amplification (LAMP), line probe assay (LPA) are the primary WHO recommended TB diagnostic methods. Some of the methods need a higher proficiency and lab setup; as a result rapid and accurate diagnosis of TB remains difficult in economically deprived regions with a high TB burden (Prakruthi & Kavitha Sunil, 2022).

In addition, the immune response to TB can be assessed using diagnostic tests that detect the presence of memory immune cells or measure cytokine release for latent TB detection. Tuberculin skin test, Interferon-Gamma Release Assays (IGRAs) and T.SPOT are the common immunological diagnostics that have been used to detect MTBC infection and differentiating active TB from latent TB (Islam et al., 2014; Lagrange et al., 2007).

1.5.3.1. Microscopic diagnosis

Sputum smear microscopy for symptomatic individuals and those at risk was endorsed in 1974 by the WHO Expert Committee on Tuberculosis (Daniel, 2006) and yet it has been in use for more than half a century globally and in Ethiopia as well.

Scientists including Neelsen, Ziehl, Ehrlich, and Rindfleisch enhanced Koch's technique identifying the bacillus through reagent and procedure modification resulting in the Ziehl-Neelsen (ZN) technique which was initially used by Ehrlich in 1882. The mordant carbolic acid (phenol) was introduced by Franz-Ziehl and the primary stain changed to basic fuchsin by Friedrich Neelsen while retaining the Ziehl's mordant in ZN staining to facilitate the penetration of the crbol fuchsin into the lipid enriched cell wall of the bacilli. Heat was employed (Hussey & Zayaitz, 2008) and named "hot staining".

The modified ZN microscopic diagnosis light emitted diode (LED) microscopy is endorsed by WHO for its increased sensitivity; however, there some study findings reported a similar sensitivity to the ZN method (Anthwal et al., 2021; Davis et al., 2013). The ZN staining method has been used for PTB patients' classification as "smear positive" or "smear negative" based on the presence or absence of acid-fast bacilli (AFB), and this classification is of particular relevance to the current study.

1.5.3.2. Rapid molecular tests (GeneXpert MTB/RIF assay)

GeneXpert (Xpert) assay was recommended by WHO in December 2010 for use in TB, multidrug-resistant TB, and TB/HIV co-infection in endemic settings to tackle the limitations of sputum smear microscopy and culture. Currently, WHO has recommended the utilization of other nucleic acid amplification tests (NAATs) including Truenat (MTB or MTB Plus) for initial diagnosis of tuberculosis (TB) for countries such as Ethiopia as well.

The highest sensitivity rate has been reported for GeneXpert in detection of adult PTB patients; however, this may be impacted by the specific MTBC strains and DR mutations detected in a particular geographic location. For instance, it had only 72% and 42% sensitivity for SPPTB and SNPTB compared to culture (Alemayehu et al., 2024) and it also falsely detected DR in significant number of patients (Akalu G et al., 2023) . Moreover, both the updated Ultra and Xpert MTB/RIF assay exhibits lower specificity in comparison to the conventional methods, culture and smear microscopy which limits its use in monitoring treatment outcome of SPPTB or bacteriologically confirmed PTB patients.

The assay uses single-use plastic cartridges with multiple chambers pre-loaded with liquid buffers and lyophilized reagent beads for sample processing, DNA extraction, and semi-nested real time-PCR (Blakemore et al., 2010). Briefly, after a specimen is transferred into the MTB/RIF cartridge and entered into the Xpert machine, all the following steps, including sample work-up, nucleic acid amplification, detection of the target sequence and result interpretation is executed automatically by starting the test on the system software.

1.5.3.3. Culture

Theodor Albrecht and Edwin Kleb tried to isolate the TB bacillus in 1867 by seeding tuberculous material on egg whites and culture in sterile flasks (Barberis et al., 2017). Culture has been the most sensitive gold standard TB detection method globally, and has facilitated the identification of all mycobacterial species including detection of those MTBC with drug resistance and NTM. However, the lengthy time and the sophisticated lab and resource requirements limit its use for routine TB diagnosis. Though both solid and liquid culture medium has been recommended by WHO, the liquid media BACTEC MGIT 960 system is superior than the solid (LJ media) due to its higher sensitivity and reduced turnaround time.

1.5.4. Overview of TB control and laboratory diagnosis in Ethiopia

TB control in Ethiopia began in the early 1960s with the establishment of TB centers and sanatoriums in 3 urban areas. In the next decade the Central Office of the National Tuberculosis Control Program was established and was succeeded by the National Tuberculosis and Leprosy Control program (NTLCP) in 1994. This harmonized TB and Leprosy coordination and management at national level. In June 2000, the NTLCP became one team within the Disease Prevention and Control Department of the ministry. Starting from 2013 this program was reorganized to incorporate regional TB/HIV programs. More recently the program has evolve to cover other lung diseases as well (MOH, 2023).

In Ethiopia it is estimated that a total of 4,000 public and 1000 private sector TB diagnostic sites are available in 2024 (MOH, 2024). About 11 regions and selected specialized hospitals provide MTBC culture facilities. The most sensitive diagnostic tests remain limited with Xpert and smear microscopy as front line methods to detect

TB (MOH, 2023). Culture is primarily indicated only for bacteriologically confirmed DR suspected patients and for phenotypic confirmation of molecularly detected DR even though more than 50% TB patients are negative by Xpert and smear microscopy (MOH, 2023, 2024).

The Ethiopian national guidelines recommend Xpert for initial screening for 648 health facilities throughout the country for sputum examination for all presumptive TB patients (MOH, 2024). However, it is not fully implemented due to limited infrastructure, skill and cartridge shortages even within the capital Addis Ababa (Mohammed et al., 2020). Therefore, patients wait more than a week for laboratory results even though alternative microscopy is available for either diagnosis or anti-TB treatment follow-up. Moreover, misclassification of patients as SNPTB based solely on clinical evaluation without laboratory testing occurs in the Country (Alemayehu et al., 2024) . In general TB laboratory diagnosis services has improved, yet more effort is needed particularly regarding SNPTB or clinically diagnosed TB that can potentially be minimized through adoption of more culture facilities.

1.6. Treatment of pulmonary tuberculosis

In 1854 Hermann Brehmer, described "TB is a curable disease" for the first time promoting treatment of many people in sanatoriums (Barbier & Wirth, 2017). The discovery of the bacteriostatic agent para-amino salicylic acid and thiosemicarbazonein and isolation of streptomycin, the first antibiotic and bactericidal agent active against MTBC used to treat TB in a young woman (Daniel, 2006; Gradmann, 2001) were important milestones in medical treatment of TB. However, the discovery of the first oral anti-mycobactericidal drug isoniazid in 1952 and rifamycins in 1957 (Barbier & Wirth, 2017) opened up a new TB treatment era that

has since been supplemented with a number of other anti-TB drugs (Table 1) (WHO, 2022b, 2022c).

Beside the treatment-based approaches the sole approved TB vaccine, Bacillus Calmette-Guerin (BCG), has been used as a preventive approach by WHO. BCG has been used more than a century and it is primarily effective in infants against TB meningitis and has insignificant protection in adults (McShane, 2011). Hence, development of a new next generation vaccine has been a major objective of global stockholders, and that in turn requires detailed investigation of the genetic basics of the MTBC and the host.

Table 1. Updated list of drugs used to treat TB and DR TB and MTBC gene mutations related to antibiotic resistance.

Drug name	**Genes mutations that confer resistance**	**Drug type**
First-line drugs		
Streptomycin (STN)	*pncA, clpC1, panD*	Aminoglycide
Isoniazid (INH)	*katG, inhA, FabG, kasA*	Analogous to ETH
Rifampicin (RIF)	*RpoB*	Rifampicin
Ethambutol (EMB)	*embB, embAB, embC*	Bacteriostatic
Pyrazinamide (PZA)*	*rpsL, gidB, murA*	Pyrazine
Second -line drugs		
Ofloxacin (OFL)	*gyrA, gyrB*	Fluoroquilone
Kanamycin (KMC)	*eis, murA*	Aminoglycide
Ethionamide (ETH)	*ethA, inhA, fabG*	Analogous to INH
Bedaquiline (BDQ)	*pepQ, Rvo678, mmpL5, mmpS5, atpE*	Diarylquinolines
Linezolid (LZD)	*RplC, rrl*	Linezolid

* can be used in MDR-TB treatment (WHO, 2022c).

1.6.1. Epidemiology of drug-resistant TB

Among the antimicrobial drug resistance (AMR) causes of death 1.5 million were related with the lower respiratory tract infection and multidrug-resistant TB (MDR-TB) is among the top five antimicrobial associated mortality (100,000-250,000) in 2019 globally (Murray et al., 2022). According to definition of WHO, MDR-TB is resistance of TB to the two first line anti-TB drugs INH and RIF; the new WHO consolidated guidelines for DR-TB treatment also included TB that is resistant to RIF (RR-TB). Pre-XDR-TB, or pre-extensively drug-resistant TB, is characterized by resistance to INH, RIF, and at least one second-line injectable medication (amikacin, capreomycin, or kanamycin) or resistance to RIF and any fluoroquinolone (such as levofloxacin or moxifloxacin). Extensively drug resistant TB, XDR-TB, is defined as resistance to INH, RIF, a fluoroquinolone (levofloxacin or moxifloxacin), and either bedaquiline or linezolid or at least one second-line injectable medication (amikacin, capreomycin, kanamycin) (WHO, 2023b).

MDR/RR-TB was responsible for nineteen thousand deaths in 2022 globally, and it was prevalent in 3.3% and 17% of newly and previously treated TB patients, respectively, which decreased by 0.06% and 3% from DR levels reported before seven years (WHO, 2023b). Ethiopia is among the 30 countries with the highest burdens of TB or TB-HIV, but is now excluded from the list of the 30 countries with the highest MDR/RR-TB burden together with Kenya and Thailand, while Zambia, Mongolia, and Nepal have joined this high burden country list (WHO, 2023b) .

There are number of study reports on the level of DR and MDR-TB in Ethiopia recent metanalysis by Reta et al., any drug resistance was 14.25% (95% confidence interval (CI): 7.05–21.44%) and 2.64% (95%CI: 1.46–3.82%) MDR-TB in newly diagnosed cases

and 11.54% (95%CI: 2.12–20.96%) respectively which depends on studied area and TB type that the highest MDR-TB was reported in Addis Ababa (Reta et al., 2022). A 21.3% and 13.4% (3% in newly diagnosed TB patients) for any DR, and MDR-TB respectively were reported by a recent multicenter study conducted. on PTB patients in different regions of Ethiopia (Yenew et al., 2024). A resistance to any drug, 23% MDR-TB 1.3% in newly diagnosed SPPTB was also reported in earlier study conducted in the country.

In Addis Ababa recently a 14% DR and 5.8% MDR-TB were reported among bacteriologically confirmed patients (Akalu G et al., 2023). Whereas, a limited published data are available on the MTB-DR level among SNPTB patients. A study report from China on newly diagnosed SNPTB reported 29.6%, 2.4% and 0.08% of the isolates were DR, MDR and extensive drug resistance (XDR) respectively (CHEN et al., 2020). An earlier institutional based study among SNPTB and SPPTB, study from Ethiopia, Addis Ababa also reported a similar proportion of DR, 25% among SPPTB and 29.8% among SNPTB while 13.5% and 8.3% MDR TB among newly diagnosed SNPTB and SPPTB patients (Desta et al., 2008). Another similar study conducted in the same area on SNPTB patients reported 8.6% MDR TB prevalence in newly diagnosed and 33% in previously treated SNPTB patients (Sinshaw et al., 2019).

All the study report results imply the equal importance of DR TB detection and monitoring and control in SNPTB like SPPTB to end TB and DR-TB. Therefore the current study aimed determining drug resistance pattern of MTBC isolates from this group of patients which will nourish the scarcely available data on this group of patients.

1.6.2. Drug resistance mechanisms of MTBC

Besides its serious global and national impacts, MDR-TB treatment has been causing a huge economic burden due to the high cost of the second-line anti-TB drugs and the long-term hospitalization of patients (Ahmad et al., 2016; Sinanovic et al., 2015). In addition, MDR-TB has a noticeable impact on psychosocial aspects of the affected population (Peddireddy, 2016). Thus, MDR–TB control requires better therapeutic and diagnostic methods, which in turn depends on a better understanding of the mechanisms of MTBC drug resistance and the mutations associated with the first-line anti-TB drugs.

There are two DR mechanisms in bacteria; a resistance that can occur through the transmission of already resistant strains, called "primary resistance'', or "acquired" de novo resistance during treatment (Garcia-Basteiro et al., 2024) (Figure 8). The de novo emergence of DR in patients can arise as a result of an insufficient drug regimen such as sub-standard dosage and quality of drug, poor adherence to treatment, and patient dependent pharmacokinetic and pharmacodynamics characteristics of the given drugs (Gumbo, Angulo-Barturen, & Ferrer-Bazaga, 2015). Moreover, a study demonstrated that prolonged continuous exposure of MTBC bacilli to lethal concentrations of an antibiotic can lead to an antibiotic persistence phase which could be a resource for the generation of genetically resistant mutants to the same antibiotic or another antibiotic (Sebastian et al., 2017).

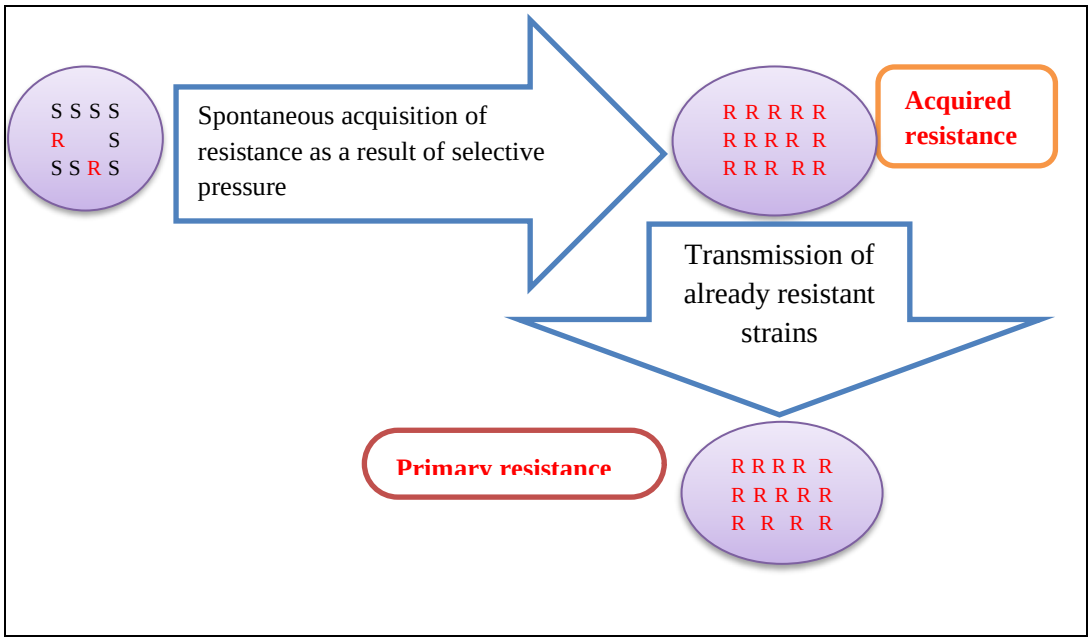


Figure 8. Diagrammatic presentation of emergence of primary and acquired drug resistance in bacteria (S: Susceptible R: resistance).

There are two distinct mechanisms of antimicrobial drug resistance; the intrinsic and acquired drug resistance. The intrinsic resistance mechanism is the inherent ability of MTBC to resist the activity of a particular antimicrobial agent through its innate structural or functional features linked to the MTBC unique cell wall properties, including the presence of mycolic acids, and β-lactamase enzymes and the efflux mechanisms that play an important role in rifampicin and isoniazid resistance (Figure 9) (Nusrath Unissa & Hanna, 2017). The Mycobacterial ability to resist a particular antimicrobial agent to which it was previously susceptible is called acquired drug resistance. Unlike the situation in other bacteria, spontaneous mutations in chromosomal genes encoding drug targets are the main mechanism in MTBC for both first and second line drugs (Nasiri, Haeili, et al., 2017).

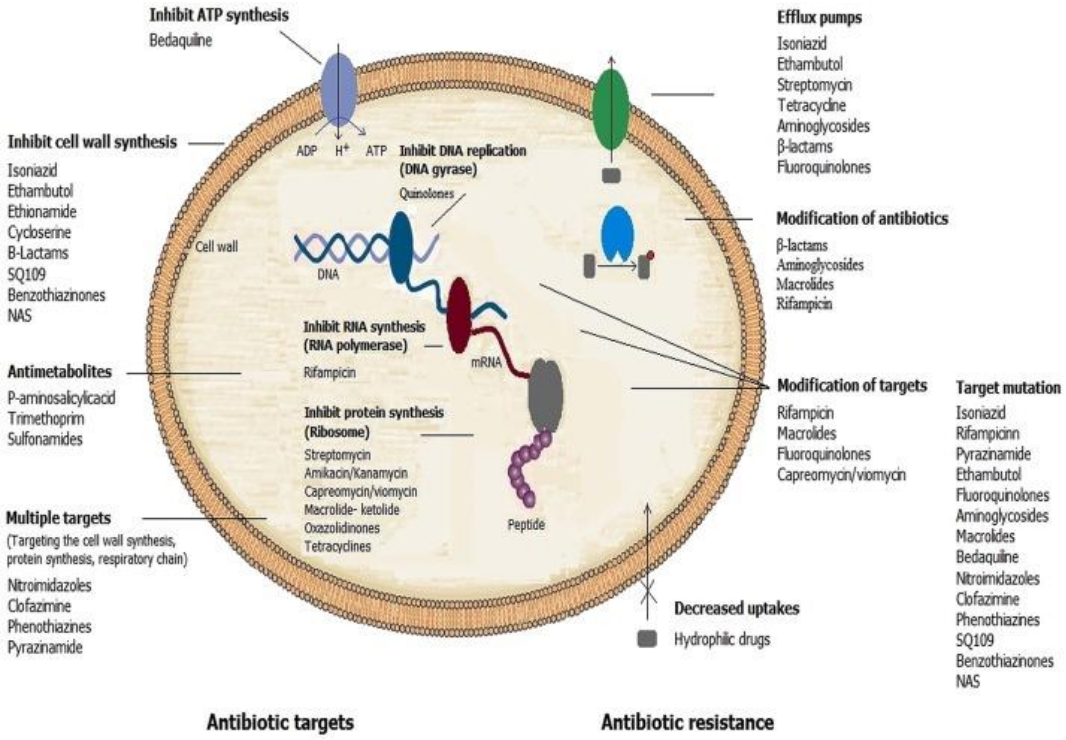


Figure 9. Mechanisms of action of antimicrobial drugs and ways by which mycobacteria become resistant to them (Nasiri et al., 2017).

In addition to the above drug resistance mechanisms, epigenetic drug tolerance may arise either from the infected host cell or infecting MTB during anti-TB drug treatment. In MTBC this has been observed frequently with latent or relapsed TB cases that may contribute to higher rates of MDR in this group of patients. For instance, epigenetic modifications in MTBC such as methylation of the 23S rRNA for resistance to second line drugs (Manderwad, 2017; Maus et al., 2005) and histone-like protein for resistance to first line anti TB drugs (Sakatos et al., 2018) enable the bacilli to alter gene expression patterns through adaptation to changing environments (J. Phelan et al., 2018; Shell et al., 2013).

The mechanisms involved to overcome the effects of first-line anti-TB drugs particularly, rifampicin the most widely used first-line anti-TB drug and the current surrogate diagnostic marker in global MDR TB control, will be discussed in detail

with further exploration on the effect of drug resistance mutations on the drug resistance detection, particularly the WHO endorsed Xpert MTB/RIF assay.

1.6.3. *rpoB* gene mutation as a primary acquired drug resistance mechanism

M. tuberculosis confers RIF resistance mainly through target alteration, i.e. mutation in *rpoB* gene which encodes the beta subunit of RNA polymerase, as well as gene mutations impacting drug cell wall permeability via efflux pumps. Furthermore, enzymatic modification of the drug is the newly identified mechanism in which a *monooxygenase* enzyme family *rifampicin monooxygenase*, inactivates rifampicin by chemically modifying its structure and confers RIF resistance (Liu et al., 2018).

1.6.4. *rpoB* gene mutations as a surrogate marker of DR-TB detection

The gene *rpoB,* encodes the beta subunit of RNA polymerase and has 1172 amino acids (Cloete et al., 2016). In MTBC the rifampicin resistance determining region (RRDR), 81-bp long (codon 433–458 or codon 507– 533 as per Escherichia coli coordinates), has been associated for RIF resistance. The most frequent mutations that confer high level of RIF resistance without any fitness loss in MTBC strains are substitutions within the codon number 441, 451 and 456 (516, 526 and 531 as per *E. coli* coordinates) (Isakova et al., 2018).

Amino acid substitutions Ser531Leu, Asp516Tyr and His 526 Asp are also common mutations conferring resistance (Figure 10). The proportion of detected mutations (usually 75-100%) within the RRDR depends on geographic location and strain type (Dookie et al., 2018; Ramasubban et al., 2015).

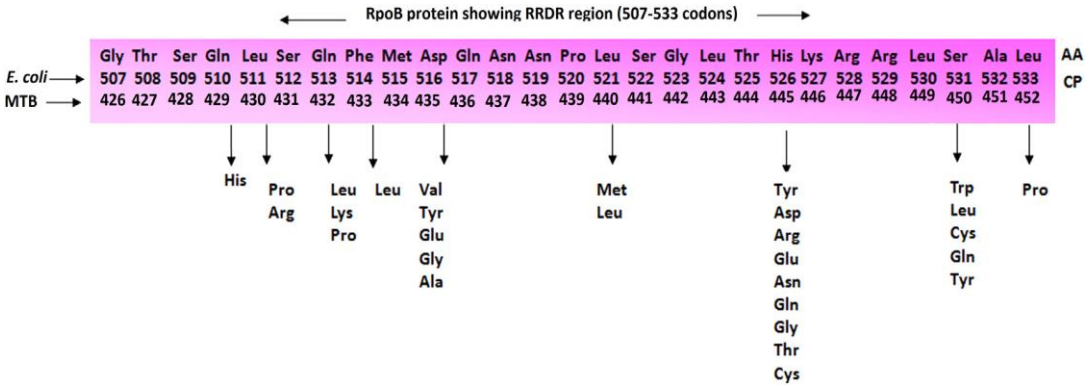


Figure 10. Most frequent mutations occur at RRDR region of RpoB proteins (Nusrath Unissa & Hanna, 2017).

Codon numbering based on *E. coli* (upper panel); codon number based on MTBC (lower panel, CP: codon position, AA: amino acid.

Molecular studies have identified novel mutations within RRDR and regions outside RRDR which are affected by strain type and geographic location. Different studies reported RIF resistant isolates with mutations at codons 471, 490, 500, 502, 503, 504, 505, 506, 507, 508, 543, 547, 553, 555, 557, 559, 562, 564 and 572 outside the RRDR (Akalu et al., 2022; Jaksuwan et al., 2017; Ramasubban et al., 2015).

Other than the alteration of RNA polymerase II activity, *rpoB* mutation contributes for intrinsic drug resistance mechanism and alters mycobacterial physiology. For instance RpoB mutation H526D is linked to a change in cell wall physiology and resistance to cell wall-related stress (Campodónico et al., 2018).

A reduced fitness of the resistant mutants as a result of acquired antibiotic resistance mechanisms affects the development stability and domination of the resistance phenotypes. WGS analysis demonstrated compensatory mutations on *rpoB* mutant MTBC isolates in the *rpoA* and *rpoC* genes. Studies reported about 47% of MDR-TB patients in TB endemic regions have mutations in either *rpoA* or *rpoC* (Casali et al., 2014). Both *rpoC* and r*poA* compensatory mutations are predominantly associated

with the predominant Ser450Leu *rpoB* gene mutation (Naidoo & Pillay, 2017; Yun et al., 2018). Enhanced fitness and transmissibility of resistant strains are related with these mutations. An *in vitro* culture study showed that resistant strains show poor growth in normal culture media without RIF (Isakova et al., 2018).

To summarize, MTBC chromosomal gene mutations particularly the RRDR of *rpoB* is considered as major mechanism of RIF resistance and is also the target for most molecular assays particularly Xpert MTB/RIF, however there are mutations outside these regions and other alternative mechanisms among drug resistant isolates which are not the target for these assays. Though, Xpert sensitivity has been improved with the newer assay (Ultra) that targets two different multi-copy genes, *rpoB* and IS6110 which allows a better detection of TB and definitive identification of RIF susceptibility and resistance (Chakravorty et al., 2017; Eldholm et al.). However, it may be inefficient for isolates with low copy number of IS6011 which is strain specific (Figure 11) (Comín et al., 2022).

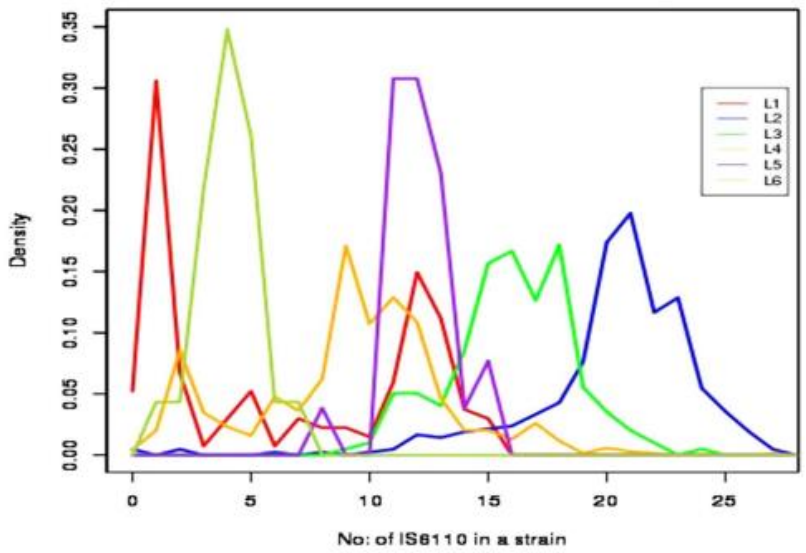


Figure 11. IS-6110 copy number distribution in lineages of MTBC.

Each lineages are denoted by a diverse colour as indicated in the figure legend (Roychowdhury et al., 2015).

Control of MDR-TB will likely require more sensitive assays allowing detection of mutants which vary according to strain and geographic area (Andre et al., 2017). To address both undetected DR-TB and false DR-TB detection, ideally WGS as a rapid test if affordable will enable patient informed TB management for DR-TB patients as well as for NTM infected individuals. Likewise, developing new molecular and non-molecular diagnostic methods (e.g. serum biomarkers) that overcome the limitation in MDR-TB diagnosis need to be identified.

1.6.5. Drug resistance detection methods

Drug resistance can be detected using either phenotypically (solid and liquid culture) which is the most accurate method even though it takes more than a week or using molecular DR detection methods which are time saving but less accurate and often need confirmation by phenotypic testing.

Prior to the start of therapy for all TB patients, with new and previous history of TB treatment, the use of the approved rapid molecular DST as the initial test to detect drug resistance is endorsed by WHO.

Currently, many programs including in Ethiopia have been carrying out DST at least for RIF at the start of TB treatment. This has been enabled by better detection through improved access for rapid molecular DR testing, particularly Xpert. In addition to the for RR testing using Xpert, LPA WHO approved a rapid polymerase chain reaction (PCR) based method designed to amplify DNA from MTBC and immobilize oligonucleotides on a strip to detect mutations commonly associated with resistance to rifampicin, isoniazid, fluoroquinolones and second-line injectable agents.

Beside its limited sensitivity, Xpert assay detect mutations within the RRDR which may not confer phenotypic or clinical resistance. Along with NAAT, Xpert tests are rapid, feasible, and accessible; however, do not identify mutations outside the *rpoB* associated gene. Conversely, WGS, at 96% concordance with culture-based drug sensitivity testing, offers a comprehensive analysis of the entire MTBC genome and genotypic sensitivity data for most drugs used in the treatment of MDR-TB. However, the full extent of the correlation between genotypic results of WGS and phenotypic sensitivities remains underexplored.

Phenotypic DR detection is yet considered as the gold standard for identifying antimicrobial susceptibilities.The modified proportion methodology works with automated liquid culture equipment like the BACTECTM MGIT 960 and requires fewer weeks than the conventional LJ culture beside its increased sensitivity for MTBC isolation. Results from sputum smear microscopy are obtained in less than 24 hours even though limited by sensitivity and DR detection as well as NTM identification from MTBC. NAAT has the ability to detect DR and has a relatively shorter turnaround time (TOT); however, such assays have higher cost, require advanced technique and higher infrastructure, and do not always correlate with phenotypic drug resistance. Culture is the most acceptable method both in sensitivity and specificity in TB and DR-TB detection, but it is limited by the time and resources. Thus, there is a continuing need for better diagnostic tools, and this requires a complete understanding of the bacterial genotype and mutations related to DR. In particular there have been few studies which address DR in SNPTB which has been viewed as a low priority.

1.7. Molecular methods used for strain typing of MTBC

Due to the low DNA sequence diversity of MTBC strains, characterization of markers like transposable and repetitive genomic elements have been attractive approaches using modern MTBC genotyping. Thus, based on the position of insertion sequence-6110 (IS-6110) throughout the genome and its variability in copy number, IS-6110-restriction fragment length polymorphism (RFLP) has been recognized as the first fingerprinting technique used to define MTBC strains (McEvoy et al., 2007). IS based typing is also used for NTM species and subspecies characterization. This includes for example *IS901, IS902, IS1245, IS900, IS901, IS902, and IS1245* used for *M. avium* species and sub-species molecular typing, and *IS6120* for *M. smegmatis* (Jagielski et al., 2014).

Subsequent to RFLP, region of differences (RD) typing, RFPLP IS610 60SrRNA analysis, spacer oligonucleotide typing (spoligotyping) and MIRU-VNTR (Supply et al., 2013) are techniques which are all based on the polymerase chain reaction (PCR) were established (Kamerbeek et al., 1997) in cascade. Currently, next generation sequencing particularly WGS has been the most powerful method for epidemiological investigation of DR and MTBC strain typing(WHO, 2023b) . In this study, spoligotyping and WGS were applied determine MTBC genetic diversity and DR which will be discussed in detail.

1.7.1. Spacer Oligo Nucleotide typing (spoligotyping)

In 1997 the PCR-based genotyping method termed spoligotyping was described and published for the first time (Kamerbeek et al., 1997). It is among the few molecular typing technique with a globally standardized database, SpolDB4 and SITVIT2 (updated version), and allows easy interpretation of results. It is also a highly sensitive

method that requires minimal genomic DNA and can be also used directly on clinical samples (Mulualem et al., 2020). Currently, it has been in use combined with Micro-satellite interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR); both use PCR based amplification of the target DNA of MTBC and WGS which targets the entire genome (Figure 12) for MTBC epidemiologic investigations. However spoligotyping is less powerful in distinguishing unrelated strains and is not suitable for NTM strain typing which can be typed by WGS (Cannas et al., 2016).

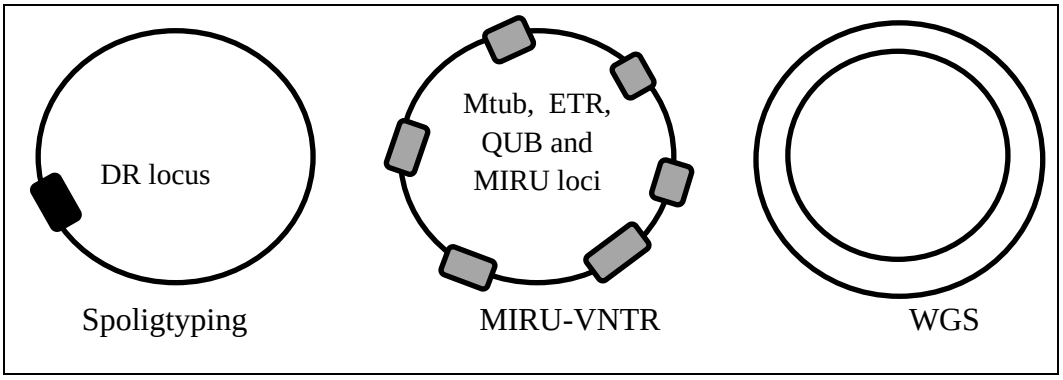


Figure 12. Diagrammatic presentation of targets of the three genotyping methods on the genome of *M. tuberculosis* H37Rv.

In spoligotyping, variation in the MTBC genome of direct spacer regions is detected. The DR locus also called the CRISPR locus and is comprised of multiple identical 36bp long regions interspersed with non-repetitive "spacer DNA sequences" that vary in length from 35bp to 41bp between each repeat as shown in Figure 13. The combination of the DR and the surrounding spacer is known as a direct variable repeat (DVR) (Kamerbeek et al., 1997).

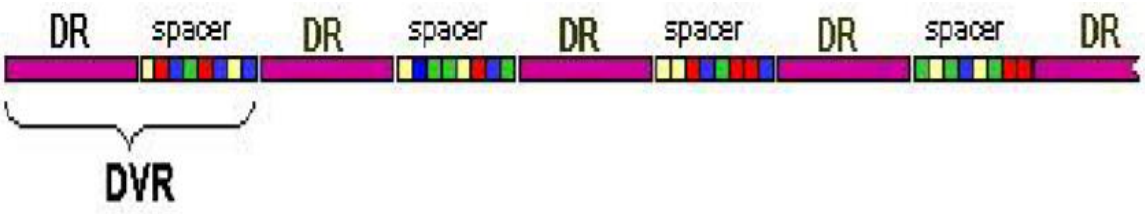


Figure 13. Diagrammatic presentation of direct variable repeat (DVR) composed of spacers and direct repeat regions (Kamerbeek et al., 1997).

The DR locus exhibits good conservation among MTBC species and currently may include up to 68 spacers, though the first recognized DVRs represented 43 distinct spacers (Kamerbeek et al., 1997). Homologous recombination between adjacent or distinct DRs as well as transformation brought by the insertion of IS-6110 results variation in DR regions (Goguet de la Salmonière et al., 1997).

Lack of a specific spacer in different MTBC strains is the base for spoligotype defined genotypes of MTBC (Kamerbeek et al., 1997). In membrane spoligotyping PCR amplification, hybridization and chemoluminesent base radiography detection are the wet lab procedures; binary code generation converted to octal code is the dry laboratory procedure. An online database containing 111,635 clinical MTBC isolates from 169 countries (Couvin et al., 2019) is continuously updated for the final prediction of MTBC strains.

The subjective base interpretation of results and consideration of homoplasy between strains; for example, a spoligotype designated as SIT4 had isolates belonging to sublineages 4.1, 4.2, 4.3, 4.4 or 4.8 (Napier et al., 2023; R. Warren et al., 2002) reveals limitation in the precise characterization of MTBC strains in spoligotyping relative to other molecular typing methods including, SNP typing assays which are used for molecular typing and DR predictions in MTBC (Stucki et al., 2012).

1.7. 2. Whole Genome Sequencing

DNA sequencing was done for the first time for the *ribonuclease* gene from *S. cerevisiae by* Holley et al and other scientists (Satam et al., 2023). Sanger's invention of the chain termination-based sequencing technique dramatically accelerated sequencing. This method produced sequence readings up to a few hundred nucleotides long by using dideoxynucleotides which stop chain elongation of DNA strands during replication (Sanger et al., 1977). Subsequently, with newer next generational sequencing approaches, it became possible to rapidly determine the entire DNA sequence of an individual's genome which is called whole genome sequencing (WGS), a potent and thorough genomic analysis method(Satam et al., 2023).

WGS makes it possible to identify genetic differences, from single-nucleotide polymorphisms (SNPs) to more significant structural alterations like insertions, deletions, and rearrangements, by sequencing every nucleotide of an organism's genome. Because WGS offers higher resolution it has been increasingly important in the epidemiological examination of MTBC. Moreover, there are tools developed for analysis of spoligotypes or other molecular markers which correlate with DNA sequence results (J. E. Phelan et al., 2019), making it the most popular molecular typing method. Most importantly, WGS has the highest discriminatory power but is more technically complex than the other molecular typing methods including spoligotyping (Cannas et al., 2016).

WGS has two types of sequencing approaches based on genome size: (1) large whole-genome sequencing, which decodes larger genomes > 5megabytes (Mb) such as from eukaryotes, and (2) small whole-genome sequencing which decodes smaller genomes of < 5Mb, mainly of prokaryotes. Short-read sequencing which was used in this study

is preferred for mutation calling, but long-read sequencing is preferred for genome assemblies (Di Marco et al., 2023).

1.8. MTBC lineage type, drug resistance and TB diagnosis

There is growing evidence that genetic diversity in MTBC contributes to the outcomes of infection and public health interventions, such as clinical disease heterogeneity, and anti-TB drug treatment outcome. For instance, epidemiological studies demonstrated that MTBC strains belonging to lineage 2 (Beijing family) was associated with adverse clinical features of TB including hyper-virulence, treatment failure, and vaccine escape (Carey, 2022). Strains of this lineage have also been reported to have greater risk for developing resistance to all first line anti-TB medications compared with other more ancient lineages (Zhou et al., 2017), suggesting the generalization that modern lineage strains may be associated with greater risk of drug resistance than ancient lineages.

The impact of MTBC strain diversity on the clinical outcome is also apparent from *M. africanum* infections which are characterized by lower rates of progression from exposure to active disease, but more severe disease among active cases compared with other MTBC strains. Such findings may also relate to geographic or host predisposition since it is primarily (but not exclusively) found in West Africa.

Similarly, MTBC lineage type has been found to be associated with disease severity by chest X-ray grading; higher frequencies of radiographic consolidation among patients infected with lineage 4 (LAM family) were observed relative to other lineages. On the other hand, patients with lineage 2 strains presented with greater numbers of cavitary lesions compared to other lineages (Peters, 2020).

In vitro growth characteristics of MTBC are also related to strain diversity. For example, MTBC lineage 4 showed faster growth rates compared to other lineages including lineage 3. Similarly, patients that harbour lineage 4 tend to have higher microscopic bacillary loads in relation to other strains such as lineage 3. Taken together, MTBC strain diversity is an important factor along with host genetics and environmental factors that impact TB clinical presentation, radiographic image findings and laboratory evaluation that are associated with clinical outcome and drug resistance. Though considerable research has been performed, basic and operational research is required for further comprehensive understanding of TB and MTBC contributing to evidence-based end TB strategy.

1.9. Statement of the problem

In Ethiopia, smear-negative TB (now referred as clinically diagnosed PTB) accounts for a significant proportion of TB cases who are often left undetected and hence leading to a significant number of morbidity and mortality (MOH, 2024). Addressing this portion contributes to better understanding of the problem, its clinical significance and molecular characterization of the causative bacterial strains. Several studies conducted in Ethiopia included molecular characterization of circulating MTBC strains that are isolated from bacteriologically confirmed PTB or SPPTB patients.

Very scarce information is available on drug resistance characteristics, and none on genetic diversity of MTBC among SNPTB patient in Ethiopia which has a higher proportions in the world of smear negative TB among all cases of pulmonary TB (WHO, 2023b). Several factors could contribute to this including limited availability of appropriate reagents and microscopes for smear tests, lack of training or technical expertise, lack of ideal antibiotics for conventional bacterial pneumonia, as well as

lack of adherence to WHO clinical algorithms for smear negative diagnosis (Mohammed et al., 2020). In addition, it is also possible that there exist unique microbiological features of the mycobacteria from smear negative cases, which preclude ease of detection, since it is known that MTBC strain diversity contributes for the outcome of laboratory diagnosis particularly, acid fast staining (AFS) bacillary load, culture positivity and abundance of cell wall lipid metabolism proteins and disease severity.

Moreover, the current available TB diagnostic tools are insufficient to achieve rapid and accurate diagnosis of TB and DR-TB resulting in empiric treatment for 38 % of the global PTB cases (MOH, 2024),. Thus, there is an urgent need for simple and highly accurate TB diagnostic technologies. The current laboratory, clinical and imaging based diagnosis of TB face certain challenges, such as delay in test result and inadequate sensitivity in sputum examination, lower specificity of geneXpert, inability to differentiate TB from other infections through imaging and clinical scoring alone due to similar manifestations, and poor specificity. Moreover, from a very limited published data, drug resistance among SNPTB was reported in a significant proportion like SPPTB while a periodic study on DR level in a given population is mandatory to monitor DR-TB. These all challenges require a periodic study on the circulating MTBC strains and understanding differences in the lineage composition of MTBC complex that could contribute for the laboratory diagnosis outcome of TB as well as MTBC drug resistance profiles in different clinical forms of TB that helps to recognize TB pathogenesis and associated diagnostic challenges for better development of therapeutics and diagnostics to end TB.

Our goals in this study were both to precisely characterize SNPTB patients according to a comprehensive algorithm of diagnostic tests, exploring the genotypes of pathogens responsible for SNPTB and determine the drug resistant profile of MTBC in SNPTB patients which is a timely research area to end TB in the Country as well as globally. The study outputs will be an impute for future improvements on the current TB diagnostic tools including smear microscopy and geneXpert test through detecting the MTBC strain difference between SNPTB and SPPTB using robust genotyping method, WGS. Moreover it will be a base for future studies on developing more accurate TB and DR-TB detecting tools. In addition, the output from drug resistance pattern will give awareness and help policy makers and TB programmers to take appropriate measures in controlling DR in SNPTB patents and the general population.

1.10. Hypothesis of the study

There is a difference in genotypes of *Mycobactrium* species and a similar drug resistance pattern between smear negative and smear positive pulmonary tuberculosis patients.

2. OBJECTIVES OF THE STUDY

2.1. General objective

- ❖ To evaluate the genetic diversity and drug resistance patterns of *Mycobacterium* species from SNPTB compared to SPPTB patients.

2.2. Specific Objectives

1. To determine the MTBC genetic diversity in SNPTB and SPPTB patients using spoligotyping and WGS.
2. To determine the phenotypic and molecular drug resistance pattern of MTBC strains isolated from SNPTB and SPPTB.
3. To determine the accuracy of smear microscopy and Xpert MTB / RIF test for the diagnosis of SNPTB compared to culture.
4. To assess the association between patient's clinical and imaging findings as well as anti-TB treatment outcomes between SNPTB and SPPTB.
5. To identify the NTM species isolated from PTB patients using Genotyping CM/AS assay and assess their clinical significance in PTB patients.

3. MATERIALS and METHODS

3.1. Study setting

The study was conducted in Addis Ababa, the capital city of Ethiopia, with an area of 527 square kilometres, a population of 46,987,000 and female comprised of 47,365,000 and male 94,352,000 in 2017 (CSA, 2017). The city is comprised of 10 sub-cities and 116 Woredas. According to the Addis Ababa Health Bureau, there are more than 700 different level private clinics, 95 public health centres, 14 public hospitals (AAHB) during the study period. A total of 155 health facilities have TB laboratory services, of which 118 have direct AFB smear microscopy test, but Xpert test and DST has been performed only in selected TB laboratory facilities through referral systems during the study period. Since 2019 Xpert MTB/RIF has been available in most TB laboratory facilities in Addis Ababa as a primary TB diagnostic service (MOH, 2023, MOH, 2024).

According to the 2017 Annual TB and Leprosy Control Program Performance Report of the Addis Ababa Health Bureau, the proportion of SPPTB SNPTB and extra-pulmonary TB (EPTB) was 26%, 26%, and 38% respectively. Concerning GeneXpert, only 17.5% of patients were positive for MBTC among those patients tested in the Xpert MTB/RIF assay.

For this study, twenty-two health centers from eight sub-cities were selected based on patient flow and availability of Xpert test laboratory as shown on the map (Figure 14) however, only were feasible and the final destination for recruitment of study participants (Figure 14).

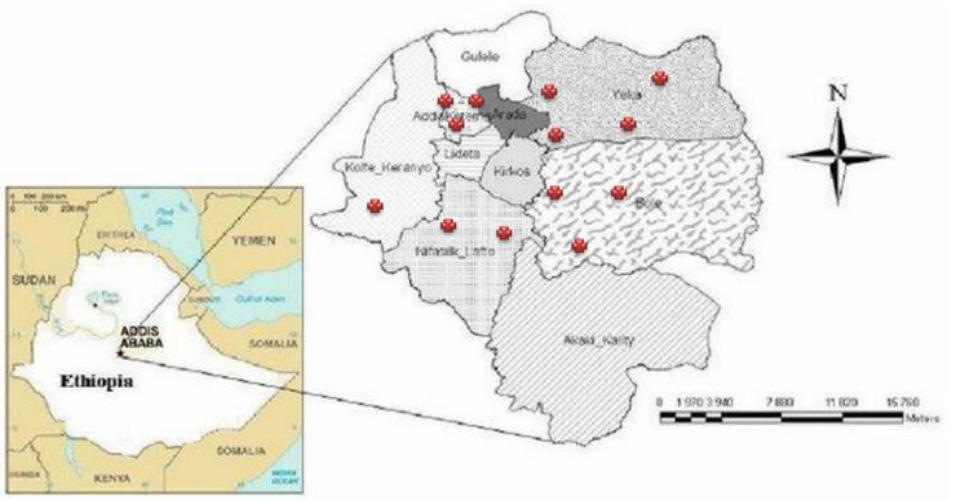


Figure 14. Map of the study area and health centres participated in the study (spotted red) modified from (Destaab & Tulubc, 2015).

Retrospectively collected and archived samples were also retrieved from the Ethiopian Public Health Institute (EPHI), which has a national TB reference laboratory and located in the capital Addis Ababa, and used for the last objectives of this study.

The study comprises four primary components: I) Microscopic, Xpert and culture analysis of sputum specimen; II) culture grown MTBC strain genotyping; III, phenotypic drug resistance determination for strains of MTBC; and IV) species typing of NTM. Each component is described in details below.

3.2. Patient recruitment procedure

All newly diagnosed and untreated presumptive PTB patients were recruited by the health personnel assigned for the study either from outpatient department (OPD) or those referred to the TB clinic of the health facility. Patients were asked to participate in the study following briefing about the study. Only patients who fulfilled the study

eligibility criteria and who signed the informed written consent were included in the study.

3.3. Study design

A health facility-based case control study with a six month follow up of patients was used for the first four objectives and cross-sectional retrospective study was conducted for the last objectives.

3.4. Sample size and sampling technique

The study was powered to according to the second aforementioned objective, to determine if clinical features differed between cases (SNPTB) and controls (SPPTB). Using the formula for an unmatched case control study built in Epi info version 3 (Dean, 2010; Jennifer L Kelsey et al., 1996), sample size was calculated assuming $\alpha = 0.05$ and $\beta = 0.8$, cough presentation (as representative clinical feature) in 71% of SPPTB and 54% of SNPTB (L. C. Campos et al., 2016) (supplementary Table 1), a 1:1 ratio of cases to controls, and over recruiting by 10% to account for incomplete data collection. With these parameters, we calculated a sample size of 153 for each group. Notably, a significant number of SNPTB patients were found to be smear positive upon retesting at the research laboratory (AHRIL) with concentrated acid-fast staining (cAFS) smear microscopy and vice versa; however, we included all the eligible participants.

All consecutive patients who visited the health facilities selected based on patient flow and availability of Xpert test during the study period were included until the required sample size was attained. For the last two objectives all the patients fulfilling the inclusion criteria were included.

3.5. Study population

Source population

The source population consisted of presumptive pulmonary tuberculosis patients diagnosed at the study sites from August 2018 through August 2021. For the last two objectives primarily PTB patients whom their sample were referred to EPHI for TB culture were source population.

Study population

Individuals who visited the selected health facilities and satisfying the Ethiopian national clinical and programmatic management of TB, leprosy and TB/HIV (EGCPMTLH) case definition of PTB were enrolled in this study. For the last two objective, the study population were PTB patient irrespective of their treatment status whom their samples were referred to EPHI for MTBC culture and NTM was recovered; primarily patients available from January 2017 to December 2017 and their data was available in the EPHI TB database were the sample population.

3.6. Inclusion and exclusion criteria

Inclusion criteria

Criteria included: newly diagnosed PTB patients, aged 18 years and above, and who were diagnosed either by smear microscopy as positive or smear negative PTB and who were prescribed with a full course of anti-TB drugs based on Xpert MTB/RIF result or clinical judgment, and willingness to sign written informed consent were enrolled.

Exclusion criteria

On the other hand, patients who were unable to produce sputum and who were initiated on anti-TB treatment for more than five days or on TB prophylaxis medication were excluded. For the last two objectives, NTM patients who had incomplete data were excluded.

3.7. Operational definitions

Definition of cases and controls

During the study period the health centers have been used Xpert, smear microscopy and/or for some patients, no laboratory testing result to diagnose PTB patients. Therefore, to keep test accuracy and uniformity patient classification was based on cAFS smear microscopy result done at Armauer Hansen Research Institute's (AHRI) research laboratory on all sputum samples. A previous study with such methodology was shown to enhance AFB detection by over 10% (Bruchfeld et al., 2000). Therefore, cases (SNPTB patients) and controls (SPPTB patients) were defined based on the cAFS microscopy analysis findings at the AHRI research laboratory.

Briefly**, SNPTB patients** were defined as patients who have been clinically diagnosed as SNPTB at health institutions based on the Ethiopian TB diagnostic algorithm (MoH, 2017) and enrolled for anti-TB treatment and later were confirmed to be AFB negative with cAFS smear microscopy analysis. Similarly**, SPPTB patients** were defined as patients diagnosed as SPPTB at health institutions based on the Ethiopian TB diagnostic algorithm (MoH, 2017) and enrolled for anti-TB treatment and were also confirmed to be AFB positive with cAFS smear microscopy analysis.

For the last two objectives on NTM study we just classified patients based on the recorded AFB result i.e., AFB positive as SPPTB and AFB negative as SNPTB.

3.8. Data collection

3.8.1. Clinical, socio-demographic and anti-TB drug treatment outcome data

Structured and pre-tested questionnaire comprised of information on socio-demographic characteristics, patient's clinical history, chest imaging findings, and specific and general predisposing factors was used; data collectors were provided training both at AHRI and on-site prior to commencement of data collection.

All patients were followed for their anti-TB drug treatment outcome for 6 months and treatment outcome were recorded according to the national guide line (MoH, 2021); 'successful' treatment outcome, if patients completed the 6 month full course of therapy or cured AFB negative sputum conversion after 6 month whereas patients classified 'unsuccessful' treatment outcome, if they have a treatment failure, death or did not complete their six month anti-TB drug treatment regiemen (MoH, 2021).

For the fifth and sixth objectives, socio-demographic, clinical and laboratory data were extracted from the EPHI's database and the patients' medical record. Briefly, based on the recorded address of referring health facilities from the EPHI database, patients who were diagnosed in hospitals located in Addis Ababa were included for further the NTM clinical importance study. Patients' medical records were critically revised using a structured data collection format and the clinical significance of the identified NTM species were determined based on the ATS/IDSA guideline (Griffith et al., 2007) that includes both clinical and microbiologic findings.

3.8.2. Specimen collection and processing

Sputum samples were collected and processed as shown in the flowchart (Figure 15). All the required safety procedures for biological specimen collection were followed as described in the global laboratory initiative biosafety manual (WHO, 2012).

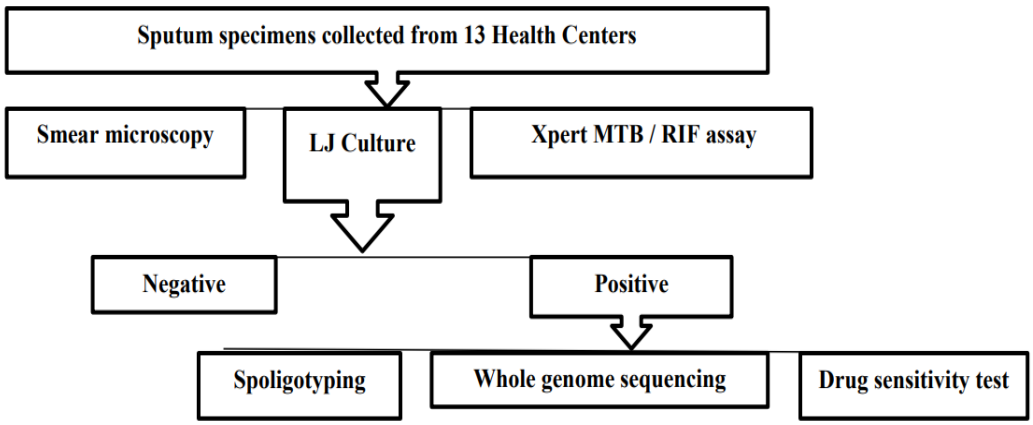


Figure 15. Flow chart for laboratory analysis of sputum specimen.

Five to ten millilitres of sputum were collected from each patient and transported to the AHRIL using a cold box. Upon arrival, samples were decontaminated using N-acetyl L-cysteine-sodium hydroxide (NALC-NaOH) and subjected to AFS, Xpert and Lowenstein-Jensen (LJ) culture and any leftover samples were refrigerated (-20°C) until further use. Xpert was performed for 109 consecutive samples according to the WHO implementation manual (WHO, 2022a). All mycobacteriology laboratory safety procedures were followed as described in the global laboratory initiative biosafety manual (WHO, 2012).

3.8.3. Microbiological analysis of sputum specimens

Smear microscopy

Acid fast staining, specifically the Ziehel Neelsen (ZN) staining method, a common method to detect mycobacteria in clinical laboratories by microscopy was applied (Hussey & Zayaitz, 2008). Briefly, the sediment of NALC-NaOH treated sputum samples were applied to glass slides, air-dried, and subsequently heat fixed. Following this, the samples underwent staining with 3% carbol fuchsin for duration of 5 minutes, after heating until vapour rise. The slides with the samples were then subjected to washing with tap water, decolourization using 3% acid alcohol, and then again washed with tap water. Finally the counter stain methylene blue was applied, the slides washed, air dried and visualized under oil immersion field (Hussey & Zayaitz, 2008).

Xpert MTB/RIF assay

MTBC complex DNA and mutations in the *rpoB* gene, which are associated with rifampicin resistance, were detected in sputum samples simultaneously by applying the following steps after sample preparation. To achieve the required sample volume for the assay, a 3:1 ratio of additional sample reagent to the NALC-NaOH decontaminated sputum pellet was employed. Manual agitation of the closed sputum container occurred twice within a 15-minute interval at room temperature. Subsequently, 2 ml of the inactivated material was then transferred to the test cartridge, equating to 0.7 ml of untreated sputum or 0.5 ml of decontaminated pellet. Following this, the cartridges were inserted into the instrument module. Finally after the instrument completed, test result were read from the screen and interpreted according to the manufacturer's instruction (Boehme et al., 2010).

Culture and identification of MTBC

LJ culture analyses were performed following a previously reported protocol (Uddin et al., 2013). Briefly, the NALC-NaOH treated sputum samples were centrifuged and the sediment was inoculated onto egg-based LJ culture medium. Two LJ medium tubes with glycerol were prepared for every sputum sample inoculation. All inoculated tubes were incubated at 37°C and the growth of MTBC was checked weekly for two months. Typical MTBC colony morphology and subsequent AFS and Capilia neo-TB test (TANUS Laboratories, Japan) were used for confirming MTBC (Arora et al., 2012).

3.8.4. Phenotypic drug sensitivity testing

The BD BACTEC MGIT 960 system and MGIT SIRE DST kit, comprised of the four first-line anti-TB drugs including, rifampicin (RIF), isoniazid (INH), ethambutol (ETH) and, streptomycin (STN) a second-line drug (WHO, 2019), was used for MTBC drug sensitivity testing according to the manufacturer instructions (Siddiqi & Rüsch-Gerdes, 2006). Briefly, LJ grown MTBC isolates were inoculated into MGIT tubes, containing the drugs at a final WHO recommended critical concentration of 1.0µg/mL for STR and RIF, 0.1µg/mL for INH, and 5µg/mL for ETH. Resistance to pyrazinamide (PZA) at 0.1µg/mL final concentration was performed using the MGIT PZA drug kit separately according to the manufacturer's instruction.

3.8.5. Molecular analysis of MTBC isolates

3.8.5.1. Spoligotyping

The spoligotyping procedure was performed according to Kamerbeek et.al. (Kamerbeek et al., 1997) using fresh heat-killed MTBC DNA. Briefly, DNA from heat killed isolates were subjected to PCR amplification after mixing with the specific

spoligotyping primers of the Direct Repeat (DR) region, DRa biotinylated at the 5' end and DRb (Thermofisher Scientific, UK) and master mix (Hot StarTaq Qiagen, Germany) and molecular grade water (Qiagen, Germany). The amplified product of each isolate was hybridized with the set of 43 immobilized oligonucleotides on biodyne-C-membrane (Animal and Plant Health Agency, United Kingdom) followed by subsequent treatment using the enhanced chemiluminescence method followed by exposure of the membrane to the X-ray film. Finally, the hybridized bands developed on the film were eye-visualized, investigated, verified, and entered into an Excel spread-sheet for further database analysis.

The verified spoligotype patterns, recorded on Excel spreadsheet, were converted into octal formats and compared with previously reported strains to define lineage, families and spoligotyping international type (SIT) (Couvin et al., 2019) using the revised online MTBC molecular marker database SITVIT2 (SITVIT2 MTBC Genotyping Database, Pasteur-guadeloupe.fr) Of note, MTBC lineage 7, which is unique to Ethiopia is predicted by spoligotyping to be *M. africanum*, also the lineage is associate with lineages 5 and 6; in contrast, WGS which is currently considered the gold standard classifies lineage 7 as *M. tuberculosis* sensu stricto.

The MTBC strains were considered 'orphans' if a single strain was found in the SITVIT2 database and 'new' if their spoligotype pattern did not match with preexisting patterns in the database. The TB-Insight online tool, Run TB-Lineage, (http://tbinsight.cs.rpi.edu/run_tb_lineage.html) in particular the conformal Bayesian network (CBN), was used to assign major lineages (Shabbeer et al., 2012), and Knowledge-Based Bayesian Networks (KBBN) (Aminian et al., 2014), to assign family or sub-family for those strains that were unidentified on the main website.

3.8.5.2. Whole genome sequencing and bioinformatics analysis

DNA extraction and sequencing

The cetyltrimethyl-ammonium bromide (CTAB) method was used (Van Soolingen et al., 1991) to extract MTBC genomic DNA from heat killed fresh MTBC isolates grown on LJ solid culture media. The quality of extracted DNA was checked by gel electrophoresis and Nano drop 2000 (Thermo Fisher, Singapore). Then it was quantified by Qubit 4.0 fluorometer (Thermo Fisher, Singapore), and subjected to library preparation using Illumina DNA library prep kit (Illumina, Inc.). Finally, libraries with qualified fragment sizes checked using Bioanalyzer 2100 (Agilent, Germany) were pooled and subjected to WGS using illumina Nextseq500 sequencer (Illumina, Singapore) with paired-end read of 150 bp capacity. All procedures were performed according to the Illumina DNA Prep protocol. (https://support.illumina.com/content/dam/illuminasupport/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-09.pdf).

Bioinformatics analysis

The quality of raw reads were checked using Fastqc tool, Fast QC (Andrews, 2010) followed by trimming poor quality base an adapters with a Trimmomatic (Bolger et al., 2014) quality score of < 20. All quality checks were combined and visualized with Multi QC v1.11 (Ewels et al., 2016) (supplementary Figure 1). Contamination check was done for all reads with Kraken 2 (Wood et al., 2019). Further analysis was performed for sequence reads $\geq$ 30bp depth coverage and samples with > 95% reference genome coverage using SAMtools (H. Li et al., 2009). Duplicated reads were identified using Picard Mark Duplicates (Boad-Institute, 2019). Burrows-Wheeler Alignment Tool (BWA) (H. Li & Durbin, 2009) was used to align the raw

paired end reads to the *M. tuberculosis* H37Rv reference genome (Gene bank accession number: NC000962.3).

Variants were called using FreeBayes (Garrison & Marth, 2012) and annotated with snpEff (Cingolani et al., 2012) for further downstream analysis. Lineage and drug resistance were predicted using TB-Profiler (Coll et al., 2015). Using *M. canettii* as root, Randomized Axelerated Maximum Likelihood (RaxML) with the general time reversal (GTR) model and 1000 bootstrap value (Stamatakis, 2006) were applied for SNP based phylogenetic reconstruction.

Lineage level strain discriminatory power of the genotyping methods were calculated using the online Hunter-Gaston Discrimination Index (HGDI) (P. R. Hunter & Gaston, 1988).

i.e., $$\mathrm{HGDI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1),$$, Where N is the total number of isolates, s is the total number of different patterns and nj is the number of isolates belonging to the j^{th} pattern.

3.8.6. Clustering analysis

Spoligotyping based MTBC strain clustering was considered if two or more strains harboring identical spoligotype pattern or SIT from different patients in the study. Similarly, recent transmission index (RTI) was calculated using the formula described previously, as the ratio of the difference between number of clustered patients and the number of clusters to the total number of patients (Tessema et al., 2013).

$$\mathrm{RTI} = \frac{\text{number of patients in cluster} - \text{number of clusters}}{\text{Total number of patients}}$$

For WGS analysis strains clustering was considered based on ≤ 12 SNPs distance whereas recent transmission index was considered if ≤ SNPs difference were detected in the sequenced strains.

3.8.7. Genotyping of stored NTM isolates

Species identification was done from stored NTM isolates using Genotype CM Ver 2.0 and Genotype AS Ver 1.0 (Hain Life science, Germany) test kits**.** NTM species were interpreted and decided according to manufacturer instructions. Briefly, DNA extraction was performed after thawing and reconstructing the stored NTM isolates. The 100µl of reconstructed colonies were diluted in 300µl distilled water and inactivated at 95°C for 20 minutes and subsequently centrifuged for 15 minutes at 10,000xg. Then, the pellets were suspended in 100µl of molecular grade water. The supernatant was discarded and immediately incubated in an ultrasonic bath for 15 minutes and again centrifuged for five minutes at 13,000xg. Finally, the supernatant DNA was transferred into a sterile tube and stored at -20°C prior to subsequent amplification. All the procedures were performed strictly following the manufacturer instruction.

DNA amplification was performed accordingly using reagents supplied with the GenoType mycobacteria CM/AS assay kit (Hain Life Scince, Germeny). A PCR thermocycler (Applied Biosystems Model 2720, USA) was used for the amplification. The hybridization and detection procedures were performed according to the manufacturer's instructions of Genotype CM/AS manually using a water bath with shaker. All the procedures were performed and results interpreted according to the manufacturer instructions (https://www.immunodiagnostic.fi/wp-

content/uploads/GenoType-CM-V2_kit-insert.pdf) and (https://www.immunodiagnostic.fi/wp-content/uploads/GenoType-AS_kit-insert.pdf).

3.9. Data processing and statistical analysis

All data from questionnaire and laboratory analysis were cleared, checked and captured onto Microsoft Access and Excel 2010 (retrospective data from TB database), Redcap software of the AHRI database and SPSS version 25 (IBM, USA) double checked and exported to Stata version 17 for analysis. Mean, frequency, and proportion ware used to summarize data. Chi-square and Fisher's exact crude odds ratio (COR) and adjusted odds ratio (AOR) or multivariable logistic regression analysis (MVA) were calculated to determine associations between outcome and predictor study variables. Variables with P-value < 0.2 in COR analysis and known risk factors irrespective of P-value were included in the MVA. In all analyses a P-value < 0.05 was considered statistically significant.

3.10. Quality control of laboratory analysis

Onsite training was given for data collectors before commencing the study. All laboratory analyses were performed according to the internal standard procedures and manufacturer instructions. Quality of LJ medium was assured by sterility checking and quality control strains *M. tuberculosis* H37Rv (ATCC 27294), *M. bovis* BCG laboratory strains and molecular grade water were used for DST and spoligotyping procedures. All AFB negative samples were double rechecked by experienced laboratory staff. All samples were processed in a Biosafety level 2 and 3 cabinet with strict adherence of safe laboratory practice as per the guidelines (Van Klingeren et al., 2007).

3.11. Ethical consideration

Before commencing the work, the research proposal was evaluated and approved by the Institutional Research Ethics Board of Addis Ababa University, College of Natural & computational Sciences (IRB/030/2017), AHRI/ALERT institutional ethical review committee (PO44/17) as well as Ethics committee of Addis Ababa city administration health bureau. Respective health facility administration was requested for official permission of support and cooperation before and during the study. All study participants provided written informed consent before enrolment to the study. An official letter of co-operation was written for EPHI, and respective health institutions and permission was obtained. Confidentiality of patient's data was maintained.

4. RESULTS

4.1. Characteristics of PTB patients

A total of 353 PTB patients were involved in this study; patients who had incomplete data for either laboratory results or questionnaires were excluded and further analysis was done for the 313 PTB patients. Men comprised 55.9% of the patients and mean age of PTB patients was 33.1±12.4 years. According to Table 2, more than 72% of the patients were in the age group of 18-45 years. Those patients who came from urban areas represented 86.1%, and among these 27.3% were working as daily labourers. Regarding education, 71.4% of the patients completed at least primary school.

Table 2. Socio-demographic characteristics SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

Study variables		**SPPTB**		**SNPTB**		**Total**	
		No	(%)	No	(%)	No	(%)
Gender	Men	85	60.7	89	51.2	174	55.6
	Women	55	39.3	84	48.8	139	44.4
Age group (Years)	18-24	39	27.7	33	19.2	72	23
	25-34	68	48.2	76	44.2	144	46
	35-44	15	10.6	25	14.5	40	12.8
	>=45	19	13.4	38	22.1	57	18.3
Residence	Urban	130	92.2	152	88.4	282	90.1
	Rural	11	7.8	20	11.6	31	9.9
Occupation	Daily labourer	45	33.3	37	22.4	82	27.3
	Farmer	10	7.4	16	9.7	26	8.7
	Student	7	5.2	11	6.7	18	6
	House wife	15	11.1	7	4.2	22	7.3
	Gov't employee	14	10.4	15	9.1	29	9.7

	Unemployed	8	5.9	18	10.9	26	8.7
Educational status	Illiterate	14	10.4	25	15.3	39	13.1
	Read and write	11	8.2	25	15.3	36	12.1
	Primary	43	32.1	50	30.7	93	31.3
	Secondary	34	25.4	32	19.6	66	22.2
	Tertiary	32	23.9	31	19	63	21
Patient HIV status	Positive	19	16.1	30	21.1	49	18.8
	Negative	99	83.9	112	78.9	211	81.2
Current alcohol use	No	91	68.9	128	77.6	219	73.7
	Yes	41	31.1	37	22.4	78	26.3

No: number, Gov't: Government

The minimum body mass index (BMI) of the patients was 12 and the maximum was 39.5. More than half of the PTB patients (58%) were diagnosed in a public health facility and 37.1% were identified in private health facilities while the remaining 4.9% were screened by health extension workers who are front line health workers in the current Ethiopian health service system.

4.2. Microbiological laboratory analysis result of SNPTB and SPPTB patient

Microbiology and molecular laboratory analysis results of the 313 PTB patients included in this study are summarized and mapped as follows (Figure 16).

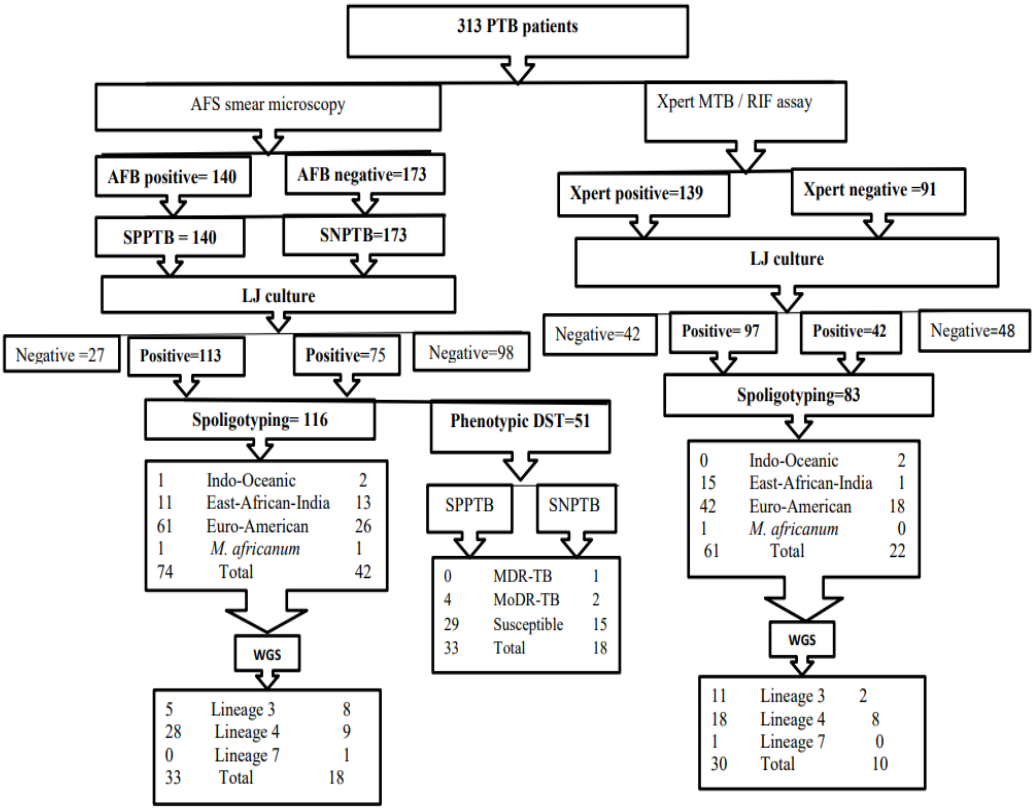


Figure 16. A flow chart mapped for microbiology and molecular analysis result of 313 PTB patients, Addis Ababa, 2021.

MoDR: Mono-drug resistant, MDR; Multidrug resistant; Pos: positive, Neg: negative.

Among the 313 PTB patients who participated in this study, 173 were SNPTB and 140 were SPPTB. Sixty-six of the SNPTB patients who were diagnosed as AFB negative at the health facilities were later found as AFB positive with cAFS smear microscopy testing at AHRIL and were reclassified as SPPTB. On the other hand, 33 patients (23 Xpert positive) who were diagnosed as SPPTB at the facilities were found to be negative following the re-examination with cAFS smear microscopy and were regrouped as SNPTB (Table 3).

Table 3. Patients' classification based on concentrated smear microscopy and clinical diagnosis (from Health Centers), Addis Ababa, Ethiopia 2021.

Classification of patients based on diagnoses at Health Centers		**Classification of patients based on cAFS smear microscopy result at AHRI laboratory**					
		SPPTB (Controls)		SNPTB (Cases)		**Total**	
		No	(%)	No	(%)	No	(%)
SPPTB	AFS	23	29.9	3	9.1	26	23.6
	Xpert	47	61.0	21	63.6	68	61.8
	Not indicated	7	9.1	9	27.3	16	12.6
	Total	77	70.0	33	30.0	110	100
SNPTB	*****Clinical and imaging findings	61	96.7	132	94.3	193	95.1
	Not indicated	2	3.2	8	5.7	10	4.9
Total		63	31.0	140	69.0	203	100

*Patients with negative laboratory result either by AFS, Xpert or classified as SNPTB without any laboratory diagnosis at the Health Centers:

A total of 230 patients had Xpert MTB/RIF test result that was done either at the health center or AHRI laboratory and 60.3% (139/230) were positive. About 38% (47/134) of SNPTB patients and 86% (92/106) of SPPTB were Xpert positive (P-value < 0.05) (Figure 17).

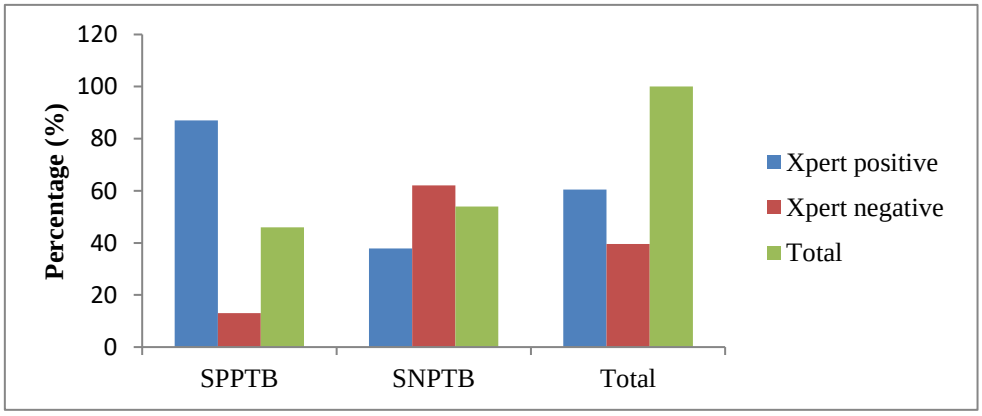


Figure 17. GeneXpert test result of smear negative PTB and smear positive PTB patients, Addis Ababa, Ethiopia 2021.

More than 40% (75/173) of SNPTB patients were LJ culture positive and that of SPPTB was 80.7% (113/140) patients (Figure 18). Xpert detected 70% (97/139) of culture positive PTB patients.

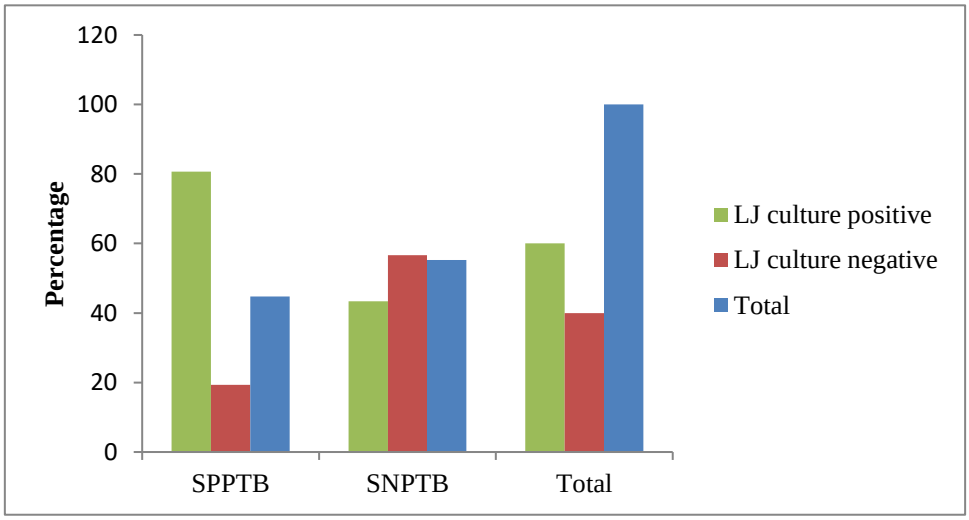


Figure 18. LJ Culture result of SNPTB and SPPTB patients Addis Ababa, Ethiopia, 2021.

4.3. MTBC genotypes among SNPTB and SPPTB patients

According to our operational definition, 173 and 140 participants were categorized as SNPTB (cases) and SPPTB (control) patients respectively. A total of 188 MTBC isolates were identified, 75 from SNPTB and 113 were from SPPTB. Of these a randomly selected 116 (74 from SPPTB and 42 from SNPTB) and 51(33 from SPPTB and 18 from SNPTB) isolates were spoligotyped and whole genome sequenced respectively.

4.3.1. Spoligotypes of MTBC isolates among SNPTB and SPPTB patients

Out the 126 spoligotyped MTBC isolates 116 (74 from SPPTB and 42 from SNPTB) had a valid result (supplementary Table 1). The overall spoligotype distribution among PTB patients consisted of the Euro-American (EA) also known as lineage 4

(75%), East-African-Indian (EAI) also known as lineage 3 (20.7%), other lineages, including Indo-Oceanic (IO) also known as lineage 1 (2.5%) and Ethiopian lineage also known as lineage 7 (identified with WGS) (1.7%). In this paper the serial lineage naming of MTBC strains is applied to align with current similar publications (Shitikov & Bespiatykh, 2023). A different MTBC lineage or clade pattern and frequency was observed between SNPTB and SPPTB patients. Both groups had predominantly strains of the lineage 4, but SPPTB had high proportions (82.4%) than SNPTB (62%), whereas isolates of the lineage 3 were more common in SNPTB (31%) compared with SPPTB (14.9%) (Table 4).

Table 4. Major spoligotype based MTBC lineages among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021

	SPPTB	**SNPTB**	**Total**	**P-value**
MTBC lineage type	No (%)	No (%)	No (%)	
Lineage 3 (East-African-Indian)	11(14.9)	13(31)	24(20.7)	
Lineage 4 (Euro-American)	61(82.4)	26(62)	87(75)	0.048
Other*	2(2.7)	3(7.1)	5(4.3)	
Total	74(63.8)	42(36.2)	116(100)	

*Other strains included; three lineage1 (Indo-Oceanic) and two lineage 7 isolate. No: number; P-value was determined by Chi-square across all MTBC lineages.

The proportion of lineage 3, lineage 4 and other lineages had a statistically significant difference among SNPTB and SPPTB (P-value < 0.05). Eleven spoligotype families were identified among all PTB patients (supplementary Table 1). MTBC strain family T, 50% (58/116) was the predominant followed by CAS, 18.1% (21/116) and Haarlem (H), 10.3% (12/116). The relative proportions of MTBC spoligotype families among SNPTB and SPPTB were 18/42 (42.9%) vs. 40/74(54%) for T and 12/42 (28.6%) vs.

9/74 (12.2%) for CAS. However, these differences did not reach statistical significance (P-value > 0.05) (Figure 19).

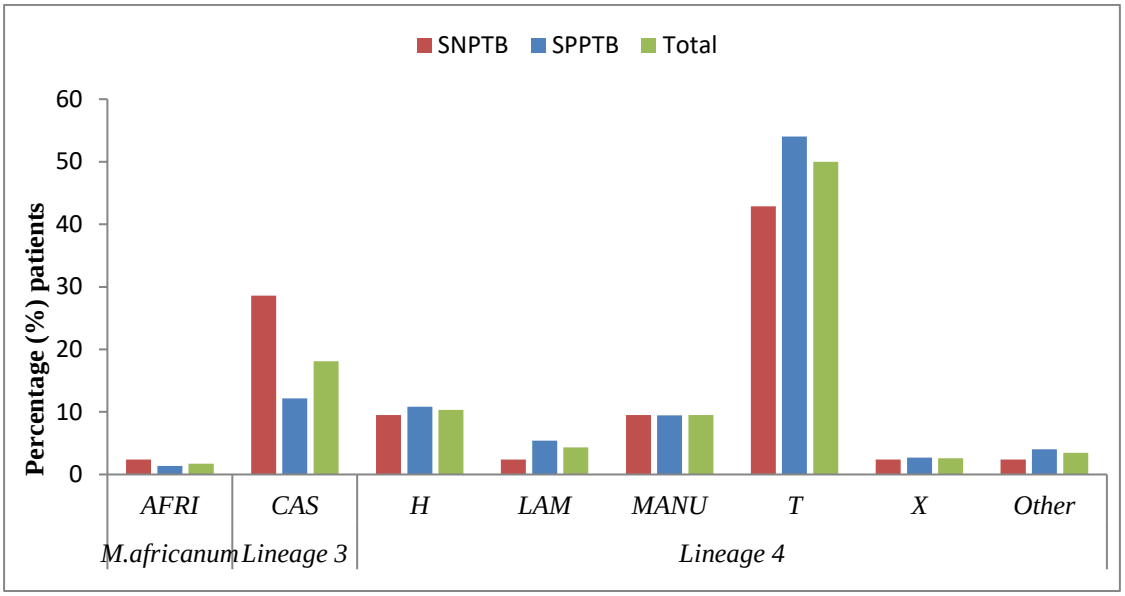


Figure 19. MTBC spoligotype family's distribution among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

* Spoligotype families/clades that include S, Zero, EAI7-BDG2, Cameroon and Unknown. SNPTB: smear negative PTB, SPPTB: smear positive PTB.

Based on analysis of shared international type (SIT) among the 116 spoligotyped MTBC strains, 84 /116 (72.4%) were pre-identified in the database, 26/116 (22.4%) were new strains, and of these 8/42 (19%) were from SNPTB and 18/74 (24%) from SPPTB. Six were orphans, four of which were from SPPTB. SIT 53 and SIT 149 were the predominant predefined strains found in this study, each comprising 11.5% (13/116) of the total spoligotyped MTBC strains (Supplementary Table 1).

In this study 82 isolates had both Xpert and spoligotyping results (61 Xpert positive and 22 Xpert negative) and the distribution of lineage 3 relative to lineage 4 differed from that seen comparing SNPTB and SPPTB. A higher proportion of lineage 4 strains were found among Xpert negative PTB patients (86.4%) than Xpert positives PTB patients (68.8%). In contrast, lineage 3 was higher among Xpert positives patients (29.5%) than among Xpert negatives PTB patients (4.6%). Two isolates of lineage 7 were identified and they were Xpert positive (P-value = 0.025) (Table 5a).

MTBC lineages were subsequently stratified according to both smear and Xpert results of PTB patients (Table 5b), and this mode of presentation clarified the differences observed when previously comparing SNPTB vs. SPPTB and Xpert negative vs. Xpert positive. Importantly, among SNPTB cases, Xpert positive and negative cases had significant differences, with the former being predominantly lineage 3 and the latter lineage 4. In contrast, among SPPTB the vast majority of cases were lineage 4 (P-value < 0.05).

Table 5a. Major Spoligotype lineages among Xpert positive and Xpert negative patients, Addis Ababa, Ethiopia 2021.

MTBC lineage type	**Xpert tests result**			
	Xpert positive	Xpert negative	Total	P-value
	No (%)	No (%)	No (%)	
Lineage 3	18 (29.5)	1 (4.8)	19 (23.2)	
Lineage 4	42 (68.8)	18 (85.7)	60 (73.2)	0.025
Other*	1 (1.6)	2 (9.5)	3 (3.7)	
Total	61 (74.4)	22 (25.6)	82 (100)	

*Other isolates included: lineage 1 (Indo-Oceanic) and lineage 7; No: number

Table 5b. Spoligotype lineage distribution with respect to GeneXpert test result among SNPTB and SPPTB patients, Addis Ababa, 2021

MTBC lineages	**SPPTB**				**SNPTB**			
	Xpert positive	Xpert negative	Total	P-value	Xpert positive	Xpert negative	Total	P-value
	No(%)	No(%)	No(%)		No(%)	No(%)	No(%)	
Lineage 3	9(18)	0	9 (16)		9(75)	10(7)	10(38)	
Lineage 4	40(82)	6(86)	46(82)	0.016	2(17)	12(86)	14(54)	0.001
Lineage 7	0	1(14)	1(2)		1(8)	1(7)	2(8)	
Total	49(88)	7(13)	56		12(46)	14(54)	26	

No: number

The spoligotype family distribution of MTBC had different proportions among positive and negative patients Xpert test results. As shown in Figure 20, T (57% vs. 43%), AFRI (4.8% vs. 1.6%) and X (4.8% vs. 1.6%) families of MTBC strains were higher among Xpert negative PTB patients than Xpert positive PTB patients. In contrast, CAS, H, S and Manu were higher among Xpert positive PTB patients than Xpert negative PTB patients. However, the overall distribution of spoligotype families did not differ significantly between the two groups (P value > 0.05) (Figure 20).

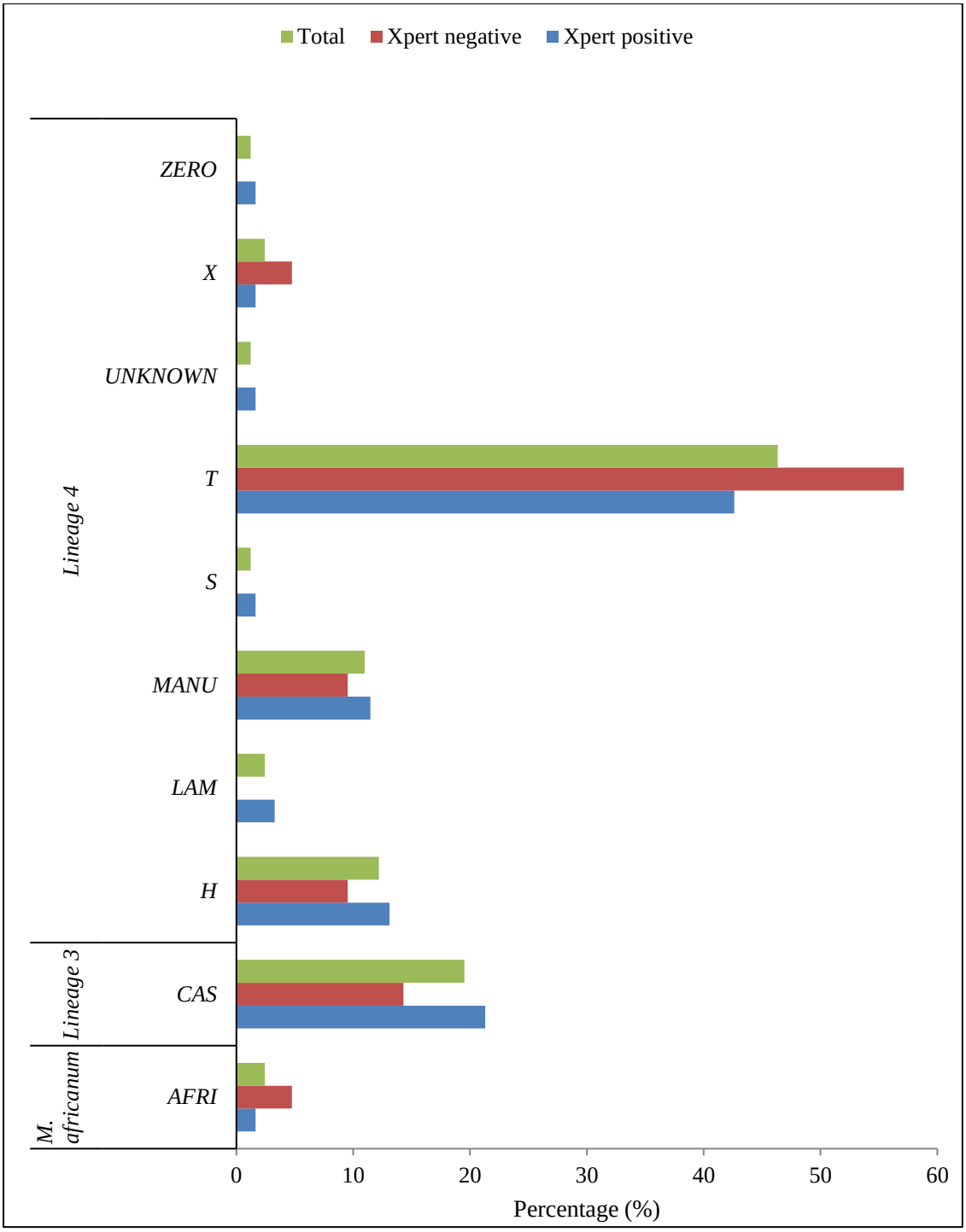


Figure 20. MTBC spoligotype families among Xpert positive and Xpert negative PTB patients, Addis Ababa, Ethiopia 2021.

4.3.2. WGS analysis of MTB strains among SNPTB and SPPTB patients

A total of 56 samples were sequenced and quality was checked including read length, G-C content, and sequence quality. Of 56, 2 were duplicates and 3 were below 65% GC content and did not passed the quality check. The quality fulfilled 51 samples were considered for downstream analysis i.e. all the samples had an acceptable sequence quality score which is greater than 30 and read length between 119bp and 138bp (supplementary Figure 1).

Among the 51 MTB isolates included in the genetic diversity and drug resistance (DR) analysis 72.5%, 25.5% and 2.0% represented lineage 4, lineage 3 and lineage 7 respectively. A different MTB lineage and sublineage pattern and frequency was observed between SNPTB and SPPTB patients mirroring the results with spoligotyping. Lineage 3 was predominant among SNPTB 44.4% (8/18) vs. 15.5% (5/33) among SPPTB (P-value = 0.056). Lineage 4 among SPPTB was 84.9% (28/33) versus 50% (9/18) in SPPTB (P-value = 0.107), shown in Table 6. The strain distribution among SPPTB and SNPTB was significantly different (P- value = 0.021) between the two groups.

Table 6. WGS based MTB lineages among SNPTB and SPPTB patients based on WGS analysis, Addis Ababa, Ethiopia 2021.

MTB lineages	SPPTB	SNPTB	Total	P-value
	No (%)	No (%)	No (%)	
Lineage 3	5(15.2)	8(44.4)	13(25.5)	
Lineage 4	28(84.9)	9 (50)	37(72.6)	0.021
Lineage 7	0	1(5.5)	1(2)	
Total	33(67.1)	18 (35.3)	51(100)	

No: number

The WGS based lineage distribution among Xpert positive and Xpert negative patients also assessed in this study. Similar to what we had observed based on spoligotype-assigned lineages; lineage 4 was proportionally higher among Xpert negative patients 8/10 (80%) than Xpert positive 18/30 (60%) (P-value < 0.05) (Table 7a). This result was different than that observed when patients were grouped into SNPTB and SPPTB. Table 7b summarizes the results of lineage associations according to smear status with additional stratification by Xpert test result. It is clear that a disproportionate number of Xpert positive SNPTB patients were evaluated by WGS and notably these patients were primarily lineage 3 associated. The Xpert stratification among SNPTB patients clearly delineates two subsets of patients, Xpert positive SNPTB patients which were primarily lineage 3 associated 6/8 (75%) and Xpert negative SNPTB patients which were primarily lineage 4 associated 6/8 (75%) (P- value = 0.037).

Table 7a. WGS based MTB lineage distribution among Xpert positive and negative PTB patients, Addis Ababa, Ethiopia 2021.

MTBC lineages	**PTB patient classification**			
	Xpert positive	Xpert Negative	Total	P-value
	No (%)	No (%)	No (%)	
Lineage 3	11(37)	2(20)	13(33)	
Lineage 4	18(60)	8(80)	26(65)	0.488
Lineage 7	1(3.3)	0	1(2.5)	
Total	30(75)	10(25)	40(100)	

No: number

Table 7a. WGS lineage distribution with respect to Xpert test result among SNPTB and SPPTB patients, Addis Ababa, 2021

MTB lineages	**SPPTB**				**SNPTB**			
	Xpert positive	Xpert negative	Total	P-value	Xpert positive	Xpert negative	Total	P-value
	No(%)	No(%)	No(%)		No(%)	No(%)	No(%)	
Lineage 3	5(28)	0	5(21)		6(75)	2(25)	8(50)	
Lineage 4	17(77)	2 (100)	19(79)	0.449	1 (13)	6 (75)	7(44)	0.037
Lineage 7	0	0	0		1 (13)	0	1(6)	
Total	22(82)	2(8)	24		8(50)	8(50)	16	

No: number

The MTB strains in the three major lineages were further subdivided as fifteen sublineages according to Figure 21 and compared between SNPTB and SPPTB patients. A different MTBC lineage and sublineage pattern and frequency was observed between SNPTB and SPPTB patients, mirroring the results with spoligotyping. Among all PTB patients, sublineage 4.2.2.2 (27.4%) was the predominant MTBC strain followed by sublineage 3 (19.6%). Sublineage comparisons between SNPTB and SPPTB are depicted in Figure 21. Sublineage 3 was more common among SNPTB (27.8%) than SPPTB (15.3%). Most of the lineage 4 sublineages were more prevalent among SPPTB than among SNPTB patients; an exception was sublineage 4.2.2.2 which had a similar distribution among SPPTB patients (27.3%) and SNPTB (27.8%) (Figure 21).

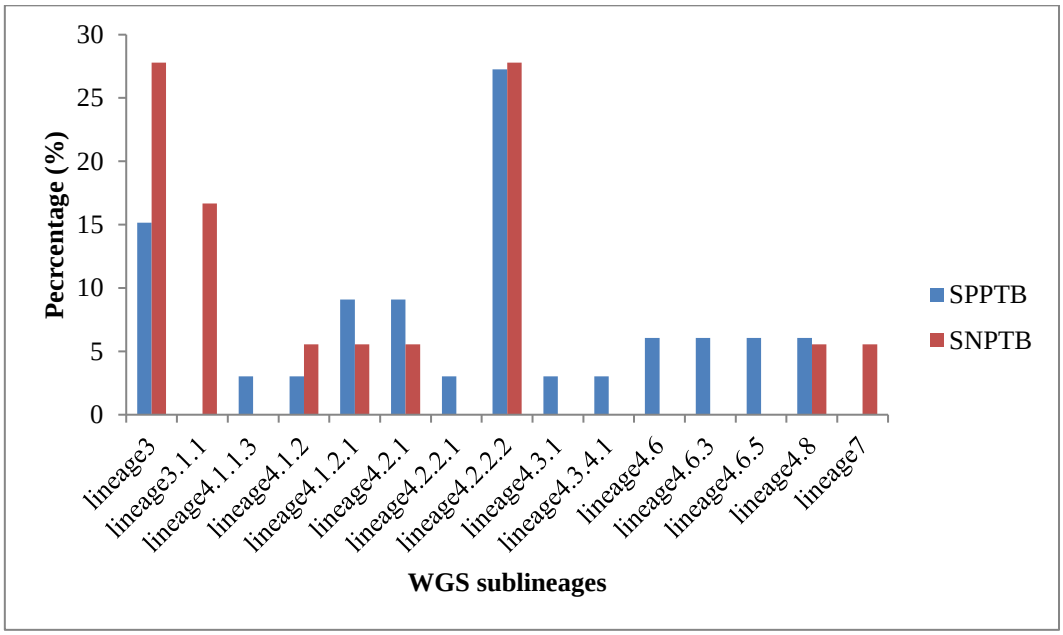


Figure 21. WGS based sublineage distribution of MTB isolates among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

4.6. Clustering analysis MTBC strains among SNPTB and SPPTB patients

4.7.1. Spoligotype based clustering analysis of strains of MTBC

Among the 116 spoligotyped isolates with 67 different patterns, 49% (61/116) were clustered with a cluster density (number of isolates per spoligotype pattern) of 2-13. The predominant strains clustered in the studied PTB population were SIT 53 and SIT 149, each with a frequency of 13 out of 69 patients (18.8%). The highest cluster density size was observed among SPPTB within the EA lineage (lineage 4), SIT 149 and SIT 53, each with a frequency of 9 out of 67 patients (Supplementary Table 1). There was no difference in the recent transmission index (a measure of the fraction of clustered patients adjusted for the number of clusters, with higher fractions resulting from lower cluster numbers presumed to reflect more recent evolution, and hence higher RTI) among SNPTB (30.9%) and among SPPTB (32.4%) (Table 8).

Table 8. Spoligotype-based MTBC strain clustering rate and recent transmission index among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

Patient type	No. of different patterns	Unique Patterns	No of clusters	No of clustered patients	Clustering Rate (%)	RTI (%)
SPPTB	50	39	11	35	47.3	32.4
SNPTB	29	24	5	18	42.8	30.9
PTB*	67#	55	14	61	49.1	40.5

*All PTB patients either SNPTB or SPPTB; # includes number of different patterns identified among PTB patients. Clustering rate is the percentage of clustered patients among total. Recent Transmission Index (RTI) = (number of clustered patients - number of clusters) / total number of patients. Isolates from SPPTB =74 and SNPTB =42.

4.7.2. WGS based clustering analysis of strains of MTB

A phylogenetic tree was constructed for the sequenced MTB isolates and lineage 4 and lineage 3 had more or less a similar clustering which was predominantly isolated from SNPTB patients (Figure 22 and Figure 23).

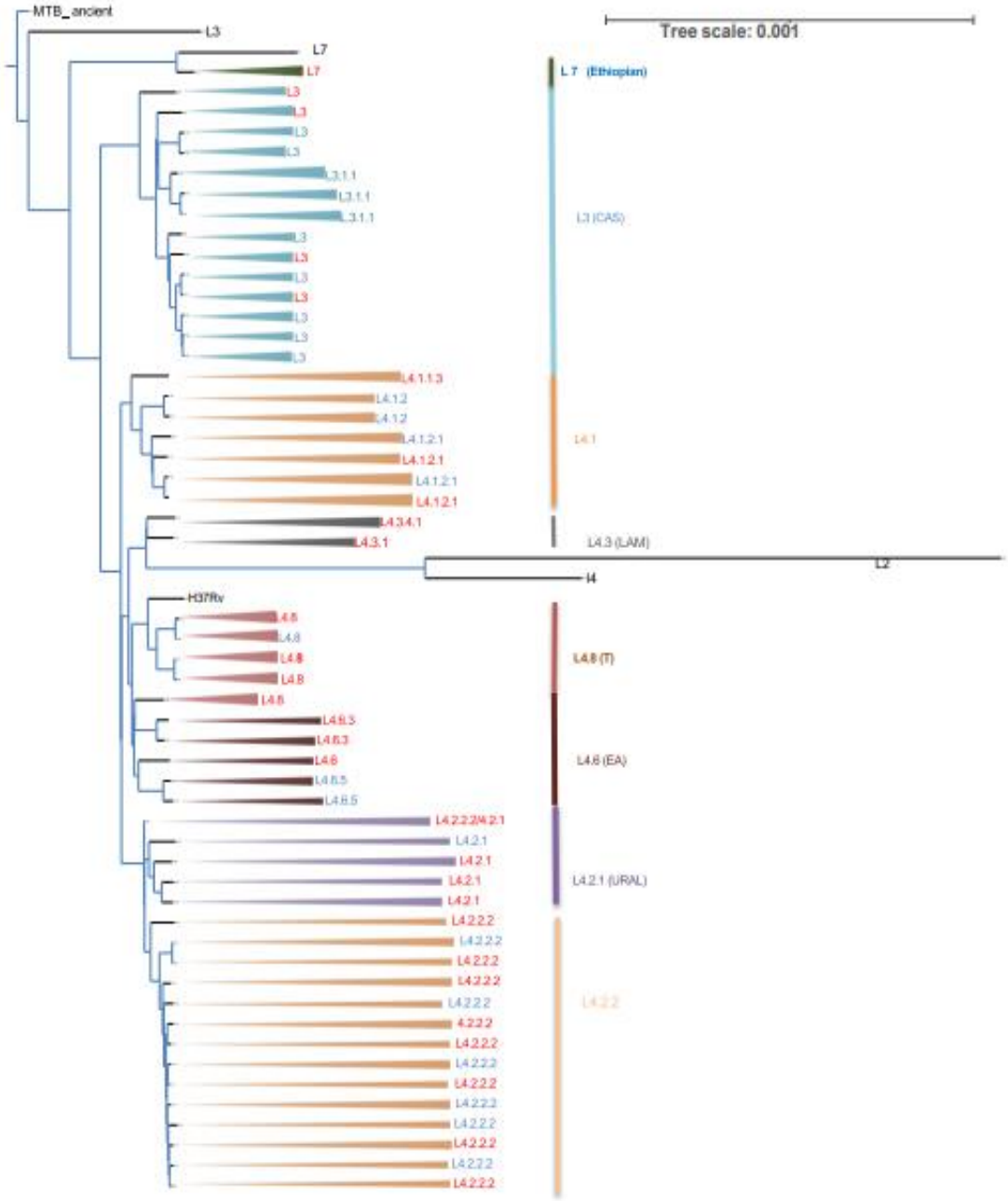


Figure 22. Phylogenetic tree of MTB strains recovered from SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

Note: Sublineage names coloured red (e.g. L4.1.1.3) are from SPPTB, blue (e.g. L3) from SNPTB, black for *M. tuberculosis* ancient *(M. canettii)* as a root for the phylogenetic tree construction. Reference genome (H37Rv) and MTB major lineages were used as a control. Randomized Axelerated Maximum Likelihood (RaxML) with the general time reversal (GTR) model and 1000 bootstrap value were used for the construction.

Of the 51 MTBC sequenced MTB strains 16% (8/51) of them were clustered (strains within a given cluster having fewer than 12 SNPs differences) (Figure 23). The WGS-based recent transmission index (where strains within a given cluster have fewer than 5 SNP differences) was about 8% (4/51).

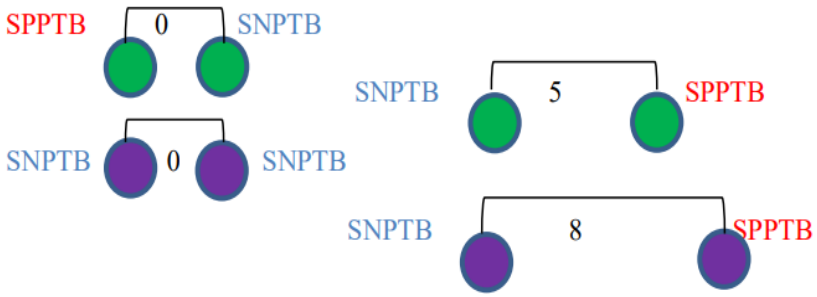


Figure 23. Clustering of MTB strains among SNPTB and SPPTB patients based on SNP difference, Addis Ababa, Ethiopia 2021.

SNPTB: smear negative PTB, SPPTB: smear positive PTB; Purple circle: lineage 3 and green circle: lineage 4, the numbers on the line indicates SNP distance.

Similar MTB lineage assignment was observed with WGS and spoligotyping with 98% concordance (44/45); except spoligotyping assigned lineage 7 as *M. africanum*. Both genotyping methods had a higher discriminatory power which was above 70%.

4.4. MTB strain drug resistance pattern among SNPTB and SPPTB patients

4.4.1. Phenotypic and molecular drug resistance in SNPTB and SPPTB patients

In the current study, phenotypic drugs resistance tests for first-line anti-TB drugs pyrazinamide (PZA), isoniazid (INH), rifampicin (RIF) and ethambutol (ETH) as well as for streptomycin (STR), were performed for 51 sequenced MTB isolates with molecular DST (WGS) result. More than 13.7% (7/51) of MTB isolates were phenotypically resistant to one or more drugs. The proportion of MTB isolates with any type of drug resistance was higher among SNPTB 16.7% (3/18) than among

SPPTB 12% (4/33) though this did not reach statistical significance (P-value > 0.05). A similar result was obtained for MDR-TB (resistance to at least RIF and INH) as shown in Table 10, but again not statistically significant. The drug resistance test for PZA was done separately for 51 isolates and three PZA resistant MTB strains including one MDRTB were detected (5.9%) and all of them were from SNPTB patients. The molecular drug resistance among PTB patients was 5.9% (3/51). Two SPPTB patients had SPPTB mono-DR (6%) while one MDR-TB was detected from SNPTB (5.6%) (P-value > 0.05) considering the composite of all drugs (Table 9).

Overall, the phenotypic DST detected 6/51 (11.8%) resistant MTB strains while molecular DST (WGS) detected 3/51 (5.9%)

Table 9. Phenotypic and molecular first-line drug (SIRE) resistance of MTB isolates among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

DST result	**Phenotypic method (MGIT)**				**Molecular method (WGS)**			
	SPPTB	SNPTB	Total	P-value	SPPTB	SNPTB	Total	P-value
	No (%)	No (%)	No (%)		No (%)	No (%)	No (%)	
MoDR	4(12)	2(11)	6(12)		2(6)	0	2(4)	
MDR	0	1(5.6)	1(2)	0.392	0	1 (5.6)	21(2)	
DS	29(88)	15(83)	44(86)		31(35)	47(65)	48(92)	0.232
Total	33 (65)	18 (35)	51		33 (65)	18 (35)	51	

MoDR: Mono-drug resistant, MDR; Multidrug resistant, DS- Drug sensitive; No: number

4.4.2. Mutations associated with drug resistance

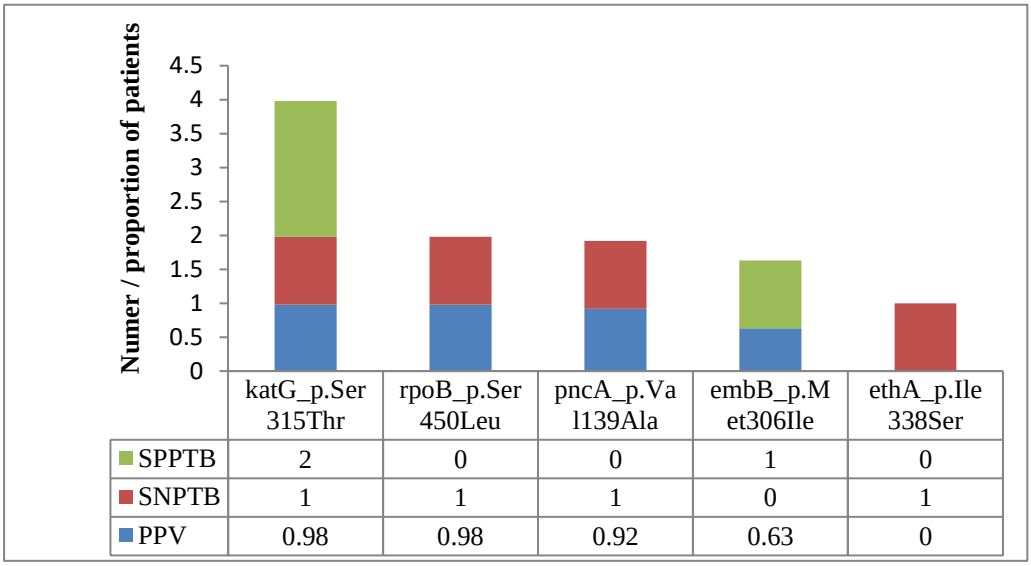
The known drug resistance mutations detected for RIF were *rpoB*_p.Ser450Leu and for INH, *katG*_p.Ser315Th. Strains with *katG* mutations had further ethanoamide drug resistance mutation *ethA_p.*Met1Arg 50% (2/3) including the MDR-TB patient

(Table 16). A new *ethA_p.*Ile338Ser mutation was detected from a SNPTB patient that was resistant to all the first-line anti-TB drugs and STN (Table 10).

Table 10. WGS based drug resistance conferring mutations detected among PTB patient, Addis Ababa, Ethiopia, 2021.

Ser. no	**Mutations detected for a particular anti-TB drugs**					
	INH	RIF	PZA	ETH	STR	Ethanoamide
1	*KatG_ p.* Ser315Thr					*ethA_p.* Met1306Arg
2	*katG_p.* Ser3154Thr	*rpoB_p.* Ser450Leu	*pncA_p.* Val139Ala	*embB_p.* Met306Ile	*rpsL_p.* Lys43Arg	ethA_p. Ile338Ser
3	*katG_p.* Ser315Thr					

All the DR mutations detected by WGS had more than 50% positive predictive value and were concordant with the updated WHO drug resistance mutation catalogue (WHO, 2023a) (Figure 24).



	katG_p.Ser315Thr	rpoB_p.Ser450Leu	pncA_p.Val139Ala	embB_p.Met306Ile	ethA_p.Ile338Ser
SPPTB	2	0	0	1	0
SNPTB	1	1	1	0	1
PPV	0.98	0.98	0.92	0.63	0

Figure 24. Drug resistance conferring mutations and their predictive value among SNPTB and SPPTB: according to the WHO 2023 DR mutation catalogue (WHO, 2023a).

A discordant result was also found in drug resistance detection by phenotypic and molecular methods. Any known mutations, including on *panA* gene were not detected by WGS for the two phenotypically PZA mono-resistant strains from SNPTB patients. Similarly mutations on *katG* gene for phenotypically INH mono-resistant strains from another two SPPTB patients were not detected. A mono-drug resistant *katG* mutation from a SPPTB patient was not detected by the phenotypic method. All the mutations are listed in the revised WHO catalogue, except the mutation for ethanoamide drug resistance (*ethA*_p.Ile338Ser) (WHO, 2023a).

4.4.3. Drug resistance and MTB strain type

The drug resistance (DR) profile of 51 MTBC strains was assessed by both the phenotypic and molecular DST methods. Out of 38 lineage 4 strains 2 were mono-drug resistant compared with lineage 3, 0% (0/13), though this was not statistically significant (P-value <0.05). A higher proportion of lineage 4 was mono-drug resistant, 2/38, than lineage 3, 0/13, whereas one multi-drug resistant lineage 3 strain from SNPTB 5.6% was detected. These differences were not statistically significant. A single strain of lineage 7 was tested and found to be drug sensitive (Table 11).

Table 11. Molecular and phenotypic drug resistance profile of 51 whole genome sequenced MTB sub-lineages, Addis Ababa, Ethiopia 2021.

WGS MTBC lineages	**Phenotypic DST**				**Molecular DST (WGS)**			
	MDR	MoDR	Sensitive	Total	MDR	MoDR	Sensitive	Total
Lineage3	1	0	9	10	1	0	9	10
Lineage3.1.1	0	0	3	3	0	0	3	3
Total (lineage 3)	**1**	**0**	**3**	**13**	**1**	**0**	**12**	**13**
Lineage4.1.1.3	0	0	1	1	0	0	1	1
Lineage4.1.2	0	0	2	2	0	0	2	2
Lineage4.1.2.1	0	1	3	4	0	0	4	4
Lineage4.2.1	0	0	4	4	0	0	4	4
Lineage4.2.2.1	0	0	1	1	0	0	1	1

Lineage4.2.2.2	0	5	9	14	0	2	12	14
Lineage4.3.1	0	0	1	1	0	0	1	1
Lineage4.3.4.1	0	0	1	1	0	0	1	1
lineage4.6	0	0	2	2	0	0	2	2
lineage4.6.3	0	0	2	2	0	0	2	2
lineage4.6.5	0	0	2	2	0	0	2	2
lineage4.8	0	0	3	3	0	0	3	3
lineage7	0	0	1	1	0	0	1	1
Total (lineage 4)	**0**	**6**	**41**	**38**	**0**	**2**	**36**	**38**

Note: Phenotypic DS: the red colored is from SNPTB and blue colored is 2 from SNPTB PZA resistant and 3 INH resistant from SPPTB; Molecular DST: the red colored is from SNPTB and blue are 2 *katG* mutations from SPPTB

4.4.4. Drug resistance and anti-TB drug treatment outcome

The anti-TB drug treatment outcomes of the 116 patients were assessed and unsuccessful treatment outcomes (failure or death) occurred in 6.9% (8/116). Unsuccessful anti-TB drug treatment outcomes were higher among culture negative SNPTB (6.7%) than culture positive SNPTB (3.5%) patients, though not of statistical significance (P-value > 0.05) (Table 12).

Table 12. Treatment outcome of SNPTB patients with respect to LJ culture, Addis Ababa, Ethiopia 2021.

Treatment outcome	**LJ culture result**							
	SNPTB				**SPPTB**			
	Positive	Negative	Total	P-value	Positive	Negative	Total	P-value
	No (%)	No (%)	No (%)		No (%)	No (%)	No(%)	
Successful	50(88)	67(94.4)	177(91)	0.182	70(90))	13(100)	83(91)	
Unsuccessful	7(12.3)	4(5.6)	11(8.6)		8(10.3)	0	8(8.8)	
Total	57(44.5)	71(55.5)	128		78(86)	13(14)	91	0.227

SNPTB: smear negative pulmonary tuberculosis: SPPTB: smear positive pulmonary tuberculosis; No: number

Of the 8 SPPTB with unsuccessful treatment outcome (including 5 deaths), 5 were initially classified as SNPTB at the health centers while among the 11 SNPTB patients (including 6 deaths), 9 were classified as SNPTB at the health centers. Based on health centers' TB patient classification the proportion of patients with

unsuccessful treatment outcomes among SPPTB patients was 6.94% (5/72) and among SNPTB was 9.52 % (14/147). Notably, the number of deaths was higher among SNPTB, 6.12 % (9/117) than SPPTB, 2.78% (2/72), though this did not reach statistical significance (P-value > 0.05).

Forty-five patients with both anti-TB drug treatment outcomes and DST result were evaluated (Table 13a); the MDR-TB patients who were SNPTB patient successfully completed the anti-TB drug treatment. Two SNPTB patients, who harbored a drug susceptible TB had an unsuccessful treatment outcome (death) (Table 13b).

Table 13. Drug resistance pattern of MTB isolates according to anti-TB drug treatment outcome of PTB patients, Addis Ababa, Ethiopia 2021.

DST result	**Treatment outcome**				
	Completed	**Cured**	**Failed/ Died**	**Total**	**P-value**
	No (%)	No (%)	No %	No (%)	
MoDR	3 (14.3)	1 (4.8)	0	4 (8.9)	
MDR	1 (4.8)	0	0	1 (2.2)	0.513
Drug Sensitive	18 (85.7)	20 (95.2)	2 (100)	40 (89)	
Total	21(50)	21 (45.7)	2 (4.4)	45 (100)	

MoDR: Mono-drug resistance, MDR; Multidrug resistance; No: number

Table 13b. Drug resistance pattern of MTB isolates according to anti-TB drug treatment outcome of PTB patients, Addis Ababa, Ethiopia 2021.

Drug sensitivity test result	**Completed or cured**		**Failed or death**		**Total**	
	SNPTB	SPPTB	SNPTB	SPPTB	SNPTB	SPPTB
MoDR	3	1	0	0	3	1
MDR	1	0	0	0	1	0
Drug Sensitive	12	37	2	0	14	37
Total	16	38	2	0	18	38

MoDR: Mono-drug resistance, MDR; Multidrug resistance; No: number

4.5. TB related clinical presentations among SNPTB and SPPTB patients

The proportion of clinical and imaging findings as well as socio-demographic characteristics of PTB patients are summarized in Table 14. Almost all TB-related symptoms were present in both SNPTB and SPPTB patients in a similar proportion; except for absence of fever, previous TB disease and normal body mass index (BMI), which were more common among SNPTB patients compared to SPPTB patients. The odds of being SNPTB among patients with previous TB was nearly three times higher than being SPPTB (P-value < 0.05) (Table 4).

Table 14. Univariate analysis of study variables among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021.

Study variables		**Health Centers' classification** SNPTB = 1; SPPTB = 0				**cAFS smear microscopy based classification** SNPTB = 1; SPPTB = 0			
		SPPTB	SNPTB			SPPTB	SNPTB		
		No(%)	No(%)	COR	P-value	No(%)	No(%)	COR	P-value
Gender	Female	49(44.6)	89(43.8)	0.97	0.905	55(39.3	83(48)	1.43	0.124
	Male	61(55.5)	114(56)	1		85(60.7)	90(52)	11	
Age group (Years)	≤45	18(16.4)	51(25.1)	1.71	0.076	22(15.7)	47(27.2)	2	0.016
	<45	92(83.6)	152(75)	1		118(84)	126(73)	11	
Occupation	Daily laborer	70(68.6)	146(75)	0.8	0.409	89(66.4)	127(77)	0.57	0.032
	Non-daily laborer	32(31.4)	50(26)	1		45(33.6)	37(22.6)	11	
Cough	No	0	8(4)	1		2(1.5)	6(3.5)	2.47	0.273
	Yes	106(96)	193(95)	1		135(98)	164(96)	11	
Weight loss	No	15(14.6)	42(21.1)	1.57	0.171	23(17)	34(20.4)	1.24	0.464
	Yes	88(85.4)	157(79)	1		112(83)	133(80)	1	
Fever	No	27(25.2)	62(31.2)	1.37	0.239	35(25.6)	54(32)	1.45	0.139
	Yes	80(74.8)	137(69)	1		102(75)	115(68)	1	
Night sweats	No	23(21.5)	41(20.8)	1		28(20.6)	36(21.4)	1	0.858

	Yes	84(78.5)	156(79)	1.04	0.889	108(79)	132(79)	0.95	
Chest pain	No	40(38.5)	63(31.7)	0.74	0.236	48(35.8)	55(32.5)	0.86	0.55
	Yes	64(61.5)	136(68)	1		86(64.2)	114(68)	1	
Shortness of breath	No	38(13)	59(20.1)	0.72	0.201	47(36.7)	50(30.3)	0.75	0.248
	Yes	62(62)	134(69)	1		81(63.3)	115(70)	1	
Hemoptysis	No	87(87)	155(82)	1.51	0.24	103(80)	139(86)	0.63	0.142
	Yes	13(13)	35(18.4)	1		26(20.2)	22(13.7)	1	
Previous TB	No	101(92)	171(84.2	1		130(93)	142(82)	1	0.007
	Yes	9(8.2)	32(15.8)	2.1	0.062	10(7.1)	31(17.9)	2.84	
HIV sero-status	Positive	17(17.7)	32(19.2)	1.14	0.673	19(15.8)	30(21)	1.29	0.39
	Negative	79(82.3)	135(81)	1		101(84)	113(79)	1	
Underweight BMI	No	119(69)	84(60)	1.3	0.282	67(60.9)	136(67)	1.47	0.106
	Yes	54(31.2)	56(40)	1		43(39.1)	67(33)	1	
BCG scar	No	56(61.5)	120(67)	1.29	0.338	78(66.7)	98(64.5)	0.91	0.708
	Yes	35(38.5)	58(32.6)	1		39(33.3)	54(35.5)	1	
Alcohol use	No	25(26)	43(23.9)	1		36(29.3)	32(20.9)	1	0.111
	Yes	71(74)	137(76)	0.89	0.693	87(70.7)	121(79)	0.64	

Further accurate analysis of the study variables for patients with a concordant classification at the health centres and cAFS smear microscopy result at AHRI research laboratory also exhibited a similar result (Table 15).

Table 15. Clinical and imaging findings of patients based on concordant classification both at the health centers and at AHRI laboratory using cAFS smear microscopy, Addis Ababa, Ethiopia 2021.

Study variables		**SPPTB**		**SNPTB**		**Total**		SNPTB=1; SPPTB = 0	
		No	%	No	%	No	%	COR	P-value
Gender	Female	31	40.3	65	46.4	96	44.2	1.29	0.382
	Male	46	59.7	75	53.6	121	55.8	1	
Age≥ 45 years	Yes	11	14.3	40	28.6	51	23.5	2.4	0.02
	No	66	85.7	100	71.4	166	76.5	1	
Daily laborer	No	24	31.6	29	21.2	53	24.9	0.58	0.094
	Yes	52	68.4	108	78.8	160	75.1	1	`_
Cough	No	0	0	6	4.3	6	2.8	1	
	Yes	75	100	133	95.7	208	97.2	1	
Weight loss	No	13	17.8	32	23.4	45	21.4	1.41	0.352
	Yes	60	82.2	105	76.6	165	78.6	1	
Fever	No	19	25	49	35.5	68	31.8	1.65	0.116
	Yes	57	75	89	64.5	146	68.2	1	
Night sweats	Yes	61	81.3	109	80.1	170	80.6	0.93	0.835
	No	14	18.7	27	19.9	41	19.4	1	
Chest pain	No	26	35.6	41	29.7	67	31.6	0.76	0.381
	Yes	47	64.4	97	70.3	144	68.3	1	
Shortness of breath	No	26	40.6	38	59.4	64	33.8	0.65	0.165
	Yes	42	30.7	95	69.3	137	66.2	1	
Hemoptysis	No	10	14.3	19	14.5	29	14.4	1.02	0.967
	Yes	60	85.7	112	85.5	172	85.6	1	

Previous TB	Yes	4	5.2	26	18.6	30	13.8	4.16	0.011
	No	73	94.8	114	81.4	187	86.2	1	
HIV sero-status	Positive	13	16.9	29	20.7	42	19.4	1.29	0.495
	Negative	64	83.1	111	79.3	175	80.7	1	
Underweight BMI	No	40	52	88	63.3	128	59.3	1.6	0.109
	Yes	37	48.1	51	36.7	88	40.7	1	
BCG scar	No	39	59.1	81	63.8	120	62.2	1.22	0.524
	Yes	27	40.9	46	36.2	73	37.8	1	
Alcohol use	yes	18	41.9	25	58.1	43	22.3	0.69	0.303
	No	50	33.3	100	66.7	150	77.7	1	

COR: crude odds ratio, BMI: Body mass index, - variables with not enough sample size to compute the statistics.

4.6. Imaging findings among SNPTB and SPPTB patients

A total of 175 patients had imaging finding results (Chest X-ray or CT scan). The proportion of patients with cavitary lesions were significantly lower among SNPTB patients, 18% (n=104) compared to SPPTB patients, 39% (n=71) (P-value < 0.05), while imaging findings other than cavitary lesions, pleural effusions or infiltration/consolidation, were four times higher among SNPTB than SPPTB patients (P-value = 0.001) (Table 16).

Multivariate analysis of selected variables showed older age, absence of fever and prior TB illness had a strong positive association with SNPTB (P-value < 0.05, whereas presence of cavitary lesions was negatively associated with SNPTB (P-value < 0.05) (Table 17).

Table 16. Univariate analysis of imaging findings among SNPTB and SPPTB, Addis Ababa, Ethiopia 2021.

Imaging outcomes		Health Center's classification				cAFS smear microscopy result classification based			
		SPPTB	SNPTB			SPPTB	SNPTB		
		No (%)	No (%)	COR	P- value	No (%)	No (%)	COR	P-value
Cavitary lesions	Yes	16(47.1)	32(21.9)	0.32	0.004	28(39.4)	20(18.3)	0.34	0.002
	No	18(52.9)	109(73)	1		43(60.6)	84(81.7)	1	
Infiltration/ consolidation	Yes	13(44.8)	43(59.4)	0.62	0.257	24(39.3)	32(33.3)	0.77	0.444
	No	16(55.2)	85(66.4)	1		37(60.7)	64(66.7)	1	
Pleural effusion	Yes	1(3)	24(17.0)	6.77	0.066	9(12.7)	16(15.4)	1.25	0.616
	No	33(76.1)	117(83)	1		62(87.3)	88(84.6)	1	
Other*	Yes	6(22.7)	44(44)	2.67	0.073	10(21.3)	39(52)	4.01	0.001
	No	16(77.3)	56(56)	1		37(78.7)	36(48.5)		

*Imaging findings other than listed in the table (includes discrete fibrosis, nodules (discrete, poorly defined), hilar lymphadenopathy, non-specific signs of pneumonia and abdominal ascites).

Table 17. Multivariate analyses of factors associated with SNPTB, Addis Ababa, Ethiopia 2021.

Study variables SNPTB=1; SPPTB = 0	**Health Center's classification**				**cAFS smear microscopy result classification based on AHRIL**				**Concordant classification both at Health center and AHRL**			
	AOR	[95% CI]		P-value	AOR	[95% CI]		P-value	AOR	[95% CI]		P-value
Female gender	0.81	0.35	1.88	0.627	1.25	0.61	2.57	0.542	1.7	0.5	5.76	0.394
Age ≥ 45 years	1.93	0.6	6.24	0.272	2.7	1.04	7.04	0.042	9.59	1.05	87.38	0.045
Non daily laborer	_	_	_	_	0.63	0.28	1.41	0.263	2.09	0.54	8.04	0.284
No weight loss	0.74	0.28	1.98	0.548	_	_	_	_	_	_	_	_
No shortness of breath	0.49	0.21	1.18	0.112	_	_	_	_	0.68	0.19	2.39	0.549
No fever	_	_	_	_	1.96	0.88	4.39	0.102	4.88	1.16	20.54	0.031
No hemoptysis	_	_	_	_	0.3	0.12	0.76	0.011	_	_	_	_
HIV positive	1.42	0.45	4.49	0.552	2.32	0.87	6.16	0.092	2.02	0.46	8.85	0.349
Previous TB	1.64	0.42	6.51	0.479	3.77	1.2	11.9	0.023	8.62	0.92	81.02	0.06
BMI ≥ 18.5	_	_	_	_	1.76	0.82	3.77	0.144	3.61	1.01	12.85	0.048
Cavitary lesions	0.35	0.13	0.92	0.034	0.33	0.14	0.77	0.011	0.26	0.08	0.86	0.027
Pleural effusion	1.38	0.45	4.19	0.573								
Other imaging findings*	_	_	_	_	2.78	0.62	12.56	0.184	1.03	0.17	6.25	0.978

*Imaging findings other than that listed in the table include discrete fibrosis, nodules (discrete, poorly defined), hilar lymphadenopathy non-specific signs of pneumonia and abdominal ascites (only 1 patient).

- Variables that did not have significant P-values in the univariate analysis

When the clinical and imaging findings of PTB patients were stratified into culture positive and culture negative patients the differences were more or less similar as when classified as SNPTB and SPPTB. Forty present (125/313) of the TB patients were diagnosed as negative on LJ culture media. Culture negative cases had an increased proportion of chest pain (73%) compared with culture positive PTB (CPPTB) (61%), and increased proportion of previous TB history (22% vs.7%). In contrast, CNPTB patients were less likely to have cavitary lesions than CPPTB patients (18.7% vs. 33%) (P-value=0.031) (Table 18). In multivariable analysis, culture negativity was significantly associated with previous TB history (P-value=0.014) and a borderline association with absence of cavitation (P-value = 0.0629) (Table 18).

Table 18. Univariate and multivariate analysis of factors associated with CNPTB, Addis Ababa, Ethiopia 2021.

Study variables CPPTB=0; CNPTB=1		**CPPTB**	**CNPTB**	**Univariate analysis**		**Multivariate analysis**			
		No (%)	No (%)	COR	P-value	AOR	[95% CI]		P-value
Gender	Female	79(42)	59(47.2)	1.2	0.366				
	Male	109(57.8)	66(52.8)	1					
Age ≥ 45 years	Yes	36(19.2)	33(26.4)	1.5	0.131	1.8	0.8	4.2	0.152
	No	152(80.5)	92(73.6)	1					
Daily laborer	Yes	57(31.3)	25(20.2)	0.5	0.032	0.4	0.2	0.8	0.018
	No	125(68.7)	99(79.8)	1					
Residence	Urban	163(87.6)	109(87)	1.0	0.910				
	Rural	23(12.4)	16(12.8)	1					
Cough	Yes	179(98.4)	120(96)	2.5	0.218				

	No	3(1.6)	5(4)	1					
Hemoptysis	Yes	146(82.9)	96(84.2)	0.9	0.779				
	No	30(17.1)	18(15.8)	1					
Chest pain	No	70(38.7)	33(27.1)	0.6	0.037	0.7	0.3	1.4	0.283
	Yes	111(61.3)	89(72.9)	1					
Night sweats	Yes	142(78)	98(80.3)	1.2	0.629				
	No	40(22)	24(19.7)	1					
Weight loss	No	29(16)	28(23.1)	1.6	0.123	1.9	0.9	4.3	0.111
	Yes	152(84)	93(76.9)	1					
Shortness of breath	Yes	116(65.5)	80(69)	0.9	0.542				
	No	61(34.5)	36(31)	1					
Previous TB	Yes	13(6.9)	28(22.4)	3.9	0.000	3.5	1.2	9.4	0.014
	No	175(93.1)	97(77.6)	1					
HIV-aerostats	Positive	23(16.4)	35(20.3)	1.1	0.804	2.2	0.9	5.1	0.081
	Negative	117(83.6)	138(79.8)	1					
BMI ≥ 18.5	Yes	64(34)	46(36.8)	0.9	0.617				
	No	124(66)	79(63)	1					
Cavitary lesion	Yes	33(33)	14(18.7)	0.5	0.036	0.5	0.2	1.1	0.065
	No	67(67)	61(81.3)						

BMI: Body mass index; No number, COR: crude odds ratio, AOR: adjusted odds ration

4.7. Association of MTBC lineages and patients clinical and imaging findings

The MTBC lineages were re-classified as either lineage 4 or non-lineage 4 and assessed for any association with the pertinent TB clinical signs and symptom and some related socio-demographic factors in the current study. While night sweats were significantly associated with lineage 4 among smear positives, a positive HIV test was also strongly associated with lineage 4 among SNPTB patients (P-value < 0.05)

(Table 19). No associations were observed between other variables including gender, residence, chest pain, hemoptysis, and cavitation and MTBC lineage type for both SNPTB and SPPTB patients (Table 19).

Table 19. Association between spoligotype based MTBC lineages and patients clinical and image findings among SNPTB and SPPTB patients, Addis Ababa, Ethiopia 2021

Study variables		**SNPTB**				**SPPTB**	
		Non-lineage 4	Lineage 4	P-value	Non-lineage 4	Lineage 4	P-value
		No (%)	No (%)		No (%)	No (%)	
Sex	Female	4(28.6)	14(50)	0.186	2(18.2)	25(39.7)	0.172
	Male	10(71.4)	14(50)		9(81.8)	38(60.3)	
Age ≥45 years	Yes	2(14.3)	10(35.7)	0.147	1(9.1)	9(14.3)	0.642
	No	12(85.7)	18(64.3)		10(90.9)	54(84)	
Daily Laborer	Yes	5(35.7)	7(28)	0.617	3(27.3)	18(29)	0.905
	No	9(64.3)	18(72)		8(72.7)	44(71)	
Cough	Yes	13(92.9)	25(100)	0.176	10(100)	62(100)	-
	No	1(7.14)	0		0	0	
Night sweats	Yes	10(71.4)	20(76.9)	0.702	5(50)	53(85.5)	0.009
	No	4(8.6)	6(23.1)		5(50)	9(14.5)	
Weight loss	Yes	3(21.4)	4(15.4)	0.631	0	13(21.7)	0.103
	No	11(78.6)	22(84.6)		10(100)	47(78.3)	
Shortness of breath	Yes	6(50)	5(21.74)	0.087	1(10)	16(27.1)	0.245
	No	6(50)	18(78.3)		9(90)	43(72.9	
Hemoptysis	Yes	0	3(11.1)	0.211	1(12.5)	11(18.3)	0.684
	No	13(100)	24(88.9)		7(87.5)	49(81.7)	
Alcohol Use	Yes	8(66.7)	24(88.9)	0.095	6(54.6)	41(74.6)	0.181
	No	4(33.3)	3(11.1)		5(45.5)	14(25.5)	
Previous TB	Yes	1(7.14)	3(10.7)	0.71	0	4(6.35)	0.39
	No	13(92.9)	25(89.3)		11(100)	59(93.7	
HIV	Negative	14(100)	19(70.4)	0.023	9(90)	48(77.4)	0.363

	Positive	0	8(29.6)		1(10)	14(22.6)	
Underweight	Yes	4(28.6)	9(33.3)	0.756	5(50)	22(35.5)	0.379
BMI	No	10(71.4)	18(66.7)		5(50)	40(64.5)	
BCG	Yes	5(62.5)	9(40.9)	0.295	4(50)	17(28.8)	0.225
vaccinated	No	3(37.5)	13(59.1)		4(50)	42(71.2)	
Cavitary	Yes	2(50)	12(28.6)	0.373	2(50)	12(28.6)	0.373
lesions	No	2(50)	30(71.4)		2(50)	30(71.4)	

No: number

4.8. Genotypes of NTM isolated from PTB patients

From January, 2017 to December, 2017, a total of 3,834 samples were processed from 698 TB patients (50.6% female and 49.4% male) with a mean age of 32.4 (±11 SD) years. Of the total, 505 (76.3%) patients had a record of their address and majority of them were from Addis Ababa City Administration 312 (63.4%) and the remaining 193 patients were from the different regions of the country: Tigray (19%), Oromia (11%), Southern Nations Nationalities and Peoples (SNNP) (2.4%) and the rest accounting for the 4.2%. These patients had been referred from outside clinicians to EPHI for further TB workup including culture and sensitivity.

4.8.1. Frequency of NTM recovered from sputum samples of PTB patients

The mycobacterial culture result of 3317 (86.5%) samples were documented, among these 401 (12%) samples from 41% (286/698) patients were positive for LJ culture (Figure 25). NTM was isolated from 60/401 (15%) culture positive samples of 51/698 (7.3%) patients; among these 6 patients had MTBC co-isolation, and 8 patients had a duplicate isolation of NTM from their samples. Of the 51 NTM culture positive patients 21 (41%) were from health facilities located in Addis Ababa City Administration.

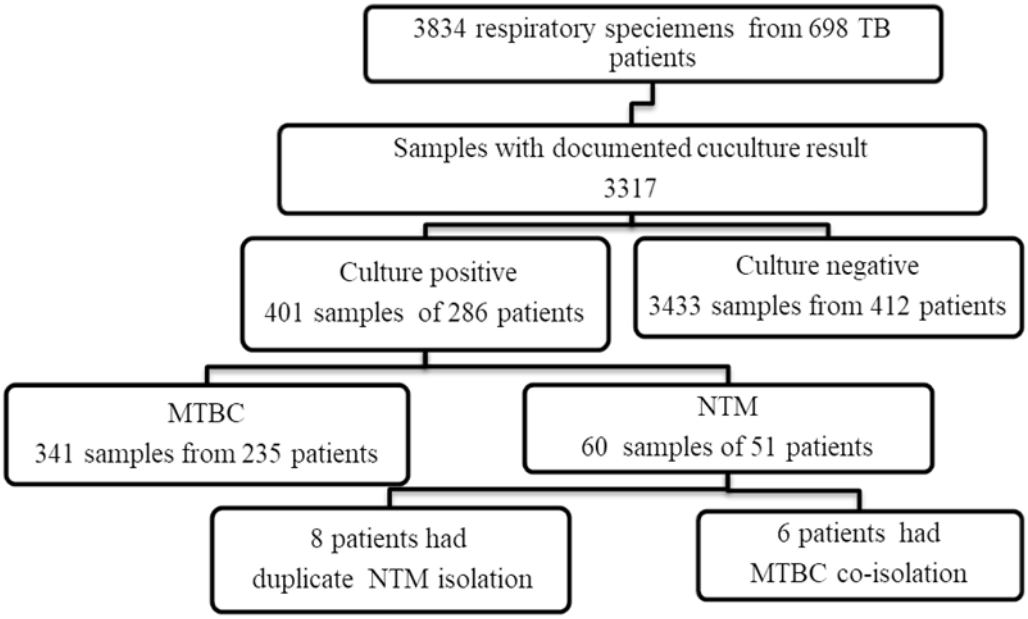


Figure 25. Flow chart for number of PTB patients and specimens identified with NTM, from January to December 2017, Addis Ababa, Ethiopia

The majority of patients from whom NTM were recovered, 42/51 (82.4%), were AFB smear negative (line 3, Table 20). The frequency of NTM isolation was determined with respect to socio-demographic and TB related factors. The proportion of NTM isolates recovered from PTB patients age group 20-29 years was the largest of all age groups, 29% (15/51) followed by age group 30-39 years old PTB patients 25% (13/51) (Table 20). Patients who had HIV positive result account for 16% (8/51); a proportion difference was observed among SPPTB (22%) and SNPTB (14%). About 26% (14/51) of NTMs were recovered from newly diagnosed PTB patients, and 22% (12/51) from relapsed PTB patients, The latter relapsed cases were higher among SNPTB 26% (11/42) compared to SPPTB (1/9). Treatment failure was higher among SPPTB patients, 33% (3/9) than among SNPTB patients, 10% (4/42) (Table 20).

Table 20. Frequency distribution NTM isolated from SNPTB and SPPTB patients, EPHI January to December 2017.

Study variables (n=51)		**SPPTB**		**SNPTB**		**Total**	
		No	%	No	%	No	%
Gender	Women	2	22	24	57	26	51
	Men	7	78	18	43	25	49
	Total	9		42		51	
	NR	0	0	2	05	2	40
Age group	<=19	2	22	8	19	10	20
	20-29	0	0	15	36	15	29
	30-39	1	11	12	29	13	25
	40-49	1	11	4	10	5	10
	50-59	2	22	1	2	3	6
	>=60	3	33	0	0	3	6
	Total	9		42		51	
HIV status	Non-reactive	4	44	11	26	15	29
	Reactive	2	22	6	14	8	16
	NR	3	33	25	60	28	55
Presumptive TB classification	New	3	33	11	26	14	27
	Relapse	1	11	11	26	12	24
	Default	0	0	1	20	1	2
	Treatment failure	3	33	4	10	7	14
	NR	2	22	15	36	17	33
Reason for test	Diagnosis	2	22	4	10	6	12
	Follow up	5	56	34	81	39	76
	NR	2	22	4	10	6	12

NR: Not recorded; No: number

4.8.2. Species of NTM isolated from PTB patients

In addition to the aforementioned NTM isolates identified from the period of January through December, 2017, additional samples were identified at EPHI from the previous years (2014-2016). From that cohort, a total of 71 stored NTM isolates were utilized for species identification including 21 isolates from patients evaluated in hospitals found in Addis Ababa and 50 isolates recovered from referral samples sent from different regions of the country. Among the 71 isolates, 25 had a valid positive result, 20 of them were NTM and 5 were MTBC with the GenoType *Mycobacterium* CM assay. The remaining 26 isolates were initially typed negative with this assay, and 18 isolates had invalid result. In a subsequent analysis of the 46 isolates typed as negative or invalid using the GenoType *Mycobacterium* CM assay, 21 had a valid result with GenoType Mycobacterium AS assay. Among these, 17 were positive for NTM, 3 were MTBC, and 1 isolate had a negative result. Overall, among the 71 genotyped probable NTM isolates only 49.3% (35/71) were NTM. Among the 28/35 patients who has the record of AFS smear microscopy result about 75% of the NTM patients had AFB negative result (SNPTB) and only 3/35 (8.7%) had an AFB positive result (SPPTB).

The NTM genotyped in this study were generally grouped into nine species, 7 from slow growers and 2 from rapid growers *at temperature 37C growth temperature.* All the 4 groups of NTMs based on Runyon classification were identified in this study (Figure 26). *M. simiae*, was the most frequently isolated slow grower NTM species 43% (15/35), followed by the rapid grower NTM, *M. abscessus*,14% (5/35), as shown in Figure 26.

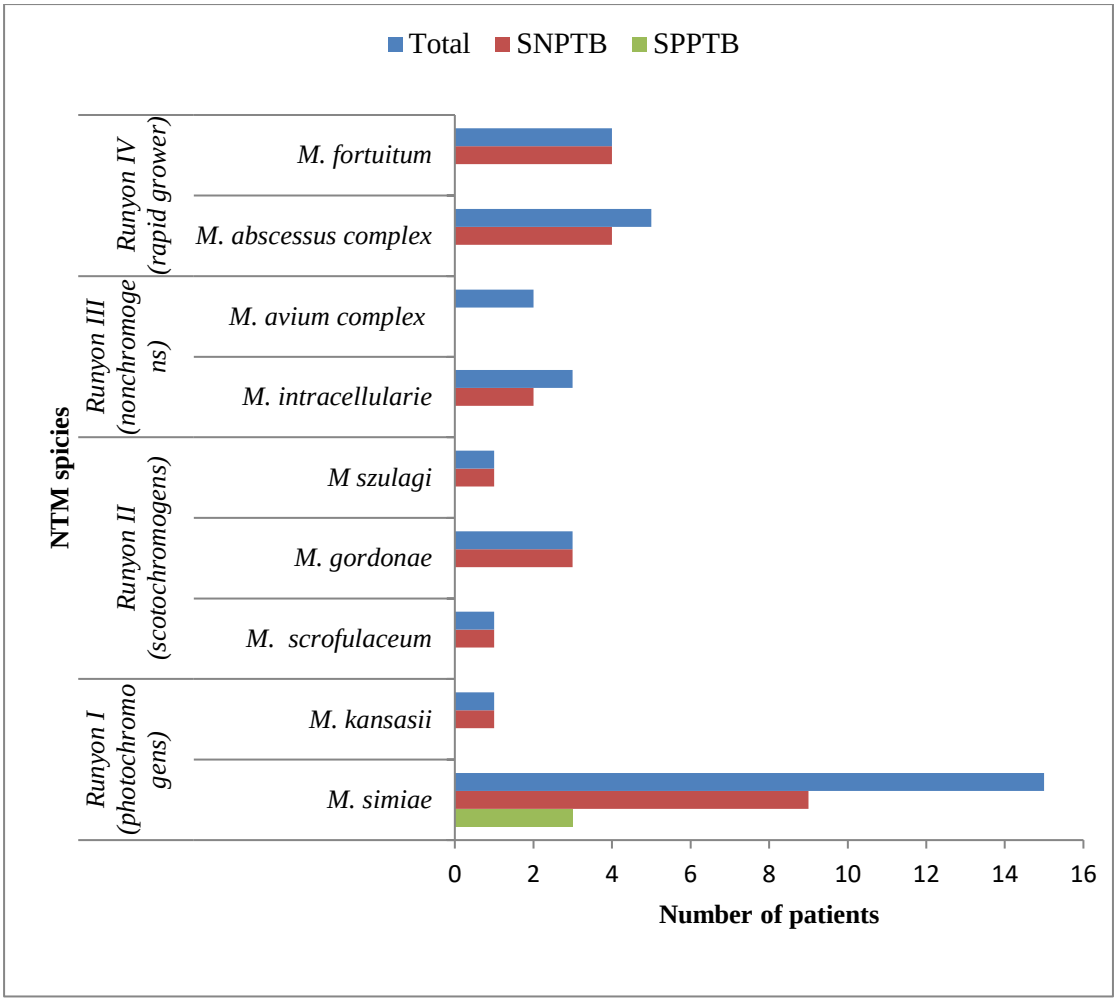


Figure 26. NTM species isolated from PTB patients at EPHI, Ethiopia 2014-2017.

4.8.3. Clinical significance of the NTM species

Fifteen patients of the 35 NTM identified from the 2016 cohort who were treated for presumptive PTB in hospitals in Addis Ababa city were further evaluated. The medical records of only 6 of these patients (40%) were available and reviewed accordingly. The medical records of 5 patients were not reviewed because of their involvement in another clinical trial study performed by WHO. Medical records of 4 patients were not found.

Based on the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) NTM disease diagnostic criteria, the NTM species identified from four

of six patients were not found to be clinically important. These patients had a favourable anti-TB drug treatment outcome and NTM was identified only from a single clinical sample after therapy. The importance of the NTM species were not determined in two patients since they did not fulfil the minimum criteria of AST/IDSA. The clinical and microbiological information of the patients are shown in Table 21. All patients were AFB negative (SNPTB), except the patient on serial number six which is AFB positive (SPPTB).

Table 21. Microbiological and clinical findings of PTB patients identified with NTM, Addis Ababa, Ethiopia 2014-2017.

Ser No	Age (years)	Reason for Test	NTM species	Cough	Chest pain	Fever	Weigh loss	Fatigue	Underlying Disease	CXR	Treatment Line	Treatment Out Come	HIV Status
1	19	New	*M. scrofulaceum*	Yes	Yes	No	Yes	No	No	NR	2nd line	Improved	NR
2	19	Treatment failure	*M szulagi*	NR	NR	NR	NR	NR	NR	NR	Both	Cured	Negative
3	70	New	*M. abscessus complex*	Yes	NR	No	Yes	NR	DM II	NR	2nd line	Improved	NR
4	22	Treatment failure	*M. fortuitum*	Yes	Yes	NR	NR	NR	NR	NR	Both	NR	NR
5	40	Treatment failure	*M. simiae*	Yes	Yes	Yes	Yes	Yes		Yes	2nd line	Improved	Negative
6	30	Relapse	*M. simiae*	Yes	Yes	NR	NR	Yes	NR	NR	NR	NR	NR

DM: diabetes mellitus, NR: not recorded

5. DISCUSSION

This study sought to evaluate the clinical, radiographic, and microbiologic characteristics of smear negative TB in Ethiopia. A unique study design was applied, in which patients were recruited based on classifications defined in standard healthcare settings, and who later were evaluated and regrouped based on the concentrated smear microscopy result performed at AHRIL. In addition, an independent cohort representing stored patients' samples sent to a mycobacterial referral at Ethiopian Public Health Institute (EPHI) was tested to provide insight into the possibility of NTM contributing to the diagnosis of TB.

Evaluation of clinical findings revealed similarities in clinical presentation with some differences among SNPTB and SPPTB patients. SNPTB were more likely to have a prior history of TB and this finding was consistent among culture negative patients compared with culture positives. This has been noted in other studies (Khadka et al., 2019; Tiamiyu et al., 2020) although not commonly reported. The underlying reason for the association between SNPTB and previous TB is not clear although it was reported that patients with SNPTB had persistently activated TB specific T cells after therapy (Esmael et al., 2022), suggesting persistence of activating mycobacterial antigens, implying a possible aetiology for future infection, yet why this would be unique to SNPTB is not clear. Alternatively, it is possible that some of the culture negative SNPTB patients with a prior history of TB had imaging findings representing old treated disease rather than active disease, with the current respiratory illness non-mycobacterial in aetiology (Mateyo et al., 2022). Further research will be required to explore these and other possible explanations.

We also observed that SNPTB patients had significantly fewer cavitary lesions as has been previously observed. The mechanism of cavitation in TB is still unclear. Whether resulting from liquefaction of caseation granulomas or following post obstructive tuberculous pneumonia, either scenario results in a large release of aerosolized bacilli in other regions of the lung and its environment (Urbanowski et al., 2020), and leading to higher bacillary loads (J. H. Kim et al., 2019). That SNPTB has substantially reduced cavitary lesions implies either a distinct mechanism of pathogenesis, or alternatively reflecting an earlier stage in the process where bacilli are less prominent.

Although SNPTB has been repeatedly shown to be more common in HIV infected individuals (Leandro Cruz Campos et al., 2016), this was not the case in our study population, where the proportion of HIV positivity was fairly similar in both groups. Another study has also reported a similar observation (Sinshaw et al., 2019). The earlier study reports on increased SNPTB in HIV may be related to a reduced cavitary disease which depends on immunocompetence (R. L. Hunter et al., 2007). Currently, the national policy of early anti-retroviral therapy initiation at high patient CD4+ counts is consistent with the notion that our cohort was probably not as immunocompromised as those of earlier studies. This may explain the lack of statistically significant difference between SNPTB and SPPTB and patients' HIV status. Consistent with this possibility, we did not see a significant difference in cavitary disease in SNPTB and SPPTB in HIV patients.

Our finding that a significant number of PTB patients initially classified as smear negative TB in health clinics, were found to be smear positive at AHRIL with concentrated smear microscopy method may partly relate to differences in individual laboratory procedures affecting sensitivity and specificity. The NALC-NaOH

procedure AHRIL uses has been shown to typically enhance AFB detection by about 10% (Tadesse et al., 2016). However, this possibility does not alone account for the discrepancies between labs, implying the contribution of additional factors.

Currently in Addis Ababa, AFB tests on smears are being phased out and replaced by Xpert for initial diagnosis for TB. Although Xpert testing is free in government clinics, it is not available in all diagnostic laboratories, and the national TB diagnosis algorithms accommodate a lack of availability of Xpert testing, indicating AFB testing as an acceptable alternative. As a result, it is common for patients to visit multiple clinics, both public and private, in the course of their diagnostic workup; thus, complex continuity of care issues may obscure diagnostic classifications. Moreover, it is possible that some clinicians confidently gave the diagnosis of TB without a smear test being done, categorizing the patient as smear negative to promote rapid treatment initiation.

Although Xpert has been recommended because of its enhanced sensitivity for TB diagnosis, some studies have shown that it fails to detect a significant percentage of culture proven TB (Kiran et al., 2022; Simieneh et al., 2022). Our findings in this study clearly confirm this, showing that only 38% of culture positive TB among SNPTB patients were Xpert positive. This percentage is lower than other reports in Ethiopia, and may reflect differences in lavatory approaches. Further studies should be done to examine in detail the actual practices of health clinics versus the recommended guidelines, and probe reasons for discrepancies.

Our finding that a significant number of patients were diagnosed as SNPTB based on clinical and radiological findings only and were negative for all microbiological tests has also been observed in previous studies (Kendall et al., 2019; Nguyen et al., 2019).

In some cases, this may reflect the simple fact that even culture is not perfectly sensitive, and may in part relate to ambiguities of obtaining consistent sputum samples. On the other hand, more comprehensive samples like Broncho alveolar lavage (BAL) and induced sputum have shown to detect over 80% of clinically diagnosed smear and Xpert negative TB (Imtiaz & Batubara, 2022). This suggests that part of the problem may be in obtaining an appropriate bacillus containing specimen by routine sputum sampling methods.

On the other hand, it is also a matter of concern that SNPTB is over diagnosed. For instance, community acquired pneumonia (CAP) elicited by bacteria not responsive to commonly used empiric antibiotics (Berhe et al., 2021) but responsive to antibiotics used in conventional or MDR TB therapy may be misdiagnosed as SNPTB. Atypical bacteria such as *Chlamydia pneumoniae* or *Mycoplasma pneumoniae* well studied in Westerns settings and characteristically treated with fluoroquinolones or macrolide antibiotics (Metlay et al., 2019) are less responsive to commonly used empiric CAP therapy in Ethiopia but potentially responsive to TB drugs like rifampicin (Kohlhoff & Hammerschlag, 2015).

Such bacteria, as well as other standard pneumonia bacteria resistant to currently used empiric therapy but responsive to TB therapy, may result in misdiagnosis of SNPTB leading to unnecessary prolonged therapy. In addition to conventional bacteria, the possibility that some diagnosed SNPTB in the clinical setting is due to non-tuberculous mycobacteria (NTM) must also be considered. While our main cohort consisted of patients recruited immediately after diagnosis, and when found to be culture positive were confirmed to be MTBC our study of a separate cohort indicated that NTM species are readily recovered in some patients, and these were disproportionally present among patients initially diagnosed with SNPTB.

This second cohort consisted of patients referred to a national diagnostic center, and most were not new cases and referred after follow-up, and over represented with treatment failure and relapse cases, thus likely not representative of our main cohort of new cases. Nonetheless, the detection of NTM in this second cohort indicates that in a typical clinic, certainly some cases of newly diagnosed SNPTB could be due to NTM, representing another means of misdiagnosis and ultimately inappropriate therapy. Therefore, further studies of SNPTB as well as investigations on other possible aetiologies should be done, including studies using more sensitive sample collection approaches such as induced sputum.

The overall lineage distribution among PTB patients is similar with other genotypic studies in Ethiopian PTB patients (Bedewi et al., 2017; Mekonnen et al., 2023; Merid et al., 2021; Worku et al., 2022) suggesting that lineage 4 and lineage 3 may have higher capacity for growth and/or fitness, are more easily transmitted, or are not easily cleared by the immune system (Osei-Wusu et al., 2021; Ramos-Martinez et al., 2019). We show here that proportionally more lineage 3 and fewer lineages 4 are found among smear negative compared with smear positive cases.

There are a number of possible explanations for this, including differences in genes related to lipid metabolism, oxidative stress or TCA cycle. For instance, there is a greater abundance of proteins involved in lipid metabolism in lineage 3 compared with lineages 4 or lineage 7 (Yimer et al., 2020). This may result in reduced penetration of the AFS reagent that precludes detection in lineage 4 isolates. However, the observed strain differences between SNPTB and SPPTB in this study will require confirmation in future experimental studies as well as studies with larger sample sizes. Moreover the higher prevalence of lineage 3 in smear negative patients

is particularly alarming since this lineage is reportedly associated with higher drug resistant lineage in the study area (Diriba et al., 2021).

The proportion of lineage 4 among SNPTB cases which were Xpert negative is higher than that of lineage 3. This could be partly associated with technical factors and sensitivity of the test which is exclusively based on the detection of *M. tuberculosis* specific *rpoB* gene and its flanking regions; however, the MTBC strain diversity may have also an impact. For example, the copy number of IS6110 varies between lineages and sub lineages; lineage 4 strains have a lower copy number of IS6110 compared to lineage 3 and other (Roychowdhury et al., 2015).

Though smear microscopy and Xpert assays utilize different approaches and differ in sensitivity, the variability of MTBC strains which is apparently dependent on the diagnostic test used, should be explored further in studies with larger sample sizes.

The predominance of MTBC spoligotypes SIT 53 and SIT 149 was similar to that reported previously both in SNPTB and SPPTB patients (Bedewi et al., 2017; Mekonnen et al., 2023; Merid et al., 2021; Worku et al., 2022). The comparable clustering rate with the recent TB transmission index among SNPTB and SPPTB detected in the current study might be partly associated with delayed TB diagnosis and treatment or may be due to the genetic makeup of MTBC strain (lineage 3) involved in SNPTB that precludes laboratory detection. However, further investigation is needed to solidify these basic findings with larger sample sizes. Ultimately, proof of differences in transmission rates will require prospective studies with molecular evaluation of both index cases and contacts.

Many early studies suggested that the transmissibility of SNPTB was considerably less than that of SPPTB, and provided justification for the focus of WHO on SPPTB

as a major means of eliminating TB. More recent studies have challenged this view, suggesting the SNPTB contributes to a greater degree of transmission than previously suggested. Many factors contribute to transmissibility, but among these include clustering capacity as well as diagnostic delay. Therefore, we were particularly interested in defining clustering index of strains, using both spoligotype and WGS approaches. With both approaches we observed similar clustering indices among SNPTB and SPPTB. Though these studies need confirmation in future studies with larger samples sizes, and in particular with studies able to directly trace person-to-person transmission, our results are in keeping with the possibility that transmission of SNPTB is greater than previously expected.

The clinical and imaging characteristics of patients with different MTBC lineages were similar among SNPTB and SPPTB, except for HIV serostatus which had a statistically significant association with MTBC lineages among SNPTB patients. This supports a study report on Ethiopian HIV patients, showing that lineage 4 is more prevalent among HIV patients than the other MTBC lineages (Chemeda et al., 2018). Moreover, reports of greater ability of lineage 4 *M. tuberculosis* in stimulating host immune response (Ramos-Martinez et al., 2019) may result in its preferential elimination among immunocompetent or HIV negative patients leading at the population level to a higher proportion of patients infected by lineage 3 bacilli. The combination of the higher proliferative capacity of lineage 4 (Osei-Wusu et al., 2021) and the compromised immune status of HIV infected individuals may result in a higher fraction of lineage 4 strains causing SNPTB in HIV positive patients.

Due to small number of isolates the data on drug resistance did not reveal statistically significant differences between SNPTB and SPPTB. There were some notable

findings; however, a patient with MDR-TB identified on the basis of culture isolates at the Armauer Hansen Research Institute research laboratory had been started on conventional anti-TB drug treatment based on clinical and sputum criteria. Failure to identify MDR-TB at the clinic level is of significant clinical relevance as it may contribute to increased transmission of these stains. These results underscore the continuing challenge of appropriate TB diagnosis.

The overall proportion of resistance to at least one of the anti-TB drug, 13.7 % (16.7% of SNPTB vs. 12% of SPPTB) during the study period was lower than a similar study conducted in Addis Ababa, Ethiopia, 30% of SNPTB vs. 25% of SPPTB (Desta et al., 2008) . Similarly our findings on MDR-TB among SNPTB patients 5.6% was similar with a another study (Damena et al., 2019) but lower from a recent study report 10% (Sinshaw et al., 2019) which both conducted in the same area. Though, these differences across the studies could be attributed to the DR detection methods (MGIT vs. LJ), study period, or method used for PTB patient classification (direct vs. concentrated smear microscopy). The reported, >5% DR prevalence implies its public health significance warranting a periodic study on MTBC drug resistance to end TB.

The current study determined PZA resistance among PTB patients and found it was higher among SNPTB patients; conversely INH resistance was higher among SPPTB patient isolates. This is the first study in Ethiopia, to our knowledge that has reported such PZA resistance among SNPTB patients; however, an increased proportion of PZA DR among SNPTB than among SPPTB was has been previously reported from China (Xia et al., 2015). Our findings warrant further study with larger sample sizes.

The most frequent gene mutations associated with INH and RIF resistance (*katG*_p. Ser3154Thr and *rpoB*_p.Ser450Leu, respectively) were similar with other study reports (Yenew et al., 2024). The newly detected *ethA*_p.Ile338Ser mutation of lineage 3 isolate from a SNPTB but Xpert MTB positive patient who was resistant for all the first line anti TB drugs implies the possibility of extensively DR-TB in this group of patients that warrants future studies including both first and second line drug resistance profile of MTBC strains with larger sample size.

Currently, DR mutations identified by WGS approaches is a research focus to improve on limitations of DST approaches based on culture and molecular methods such as GeneXpert assay. In our study all the mutations for multidrug resistant MTBC isolate identified by WGS which have reportedly greater than 50% positive predictive value indeed agreed with the phenotypic DR test, arguing for universal DST testing on culture positive isolates particularly for clinically diagnosed or SNPTB patients. However, among strains with defined phenotypic resistance, some isolates had no defined mutations by WGS within resistance genes we evaluated. This could be due to resistance genes such as efflux pumps, or mutations in cell wall proteins, but we did not look for these possibilities (Jones et al., 2022). Similar findings were also reported by a recent study (Naluyange et al., 2020). Rifampicin resistance of one MDR TB isolate detected by both phenotypic and WGS methods was undetected by Xpert in the current study, similar to the recent study reports from and Tanzania and Ethiopia (Alemu et al., 2020; Torokaa et al., 2024). These could be associated with the type of sputum specimen (fresh vs. stored; processed vs. raw) (Steingart et al., 2014), low amount of DNA in the sputum specimens (Jean Claude S Ngabonziza et al., 2021; Ocheretina et al., 2016).

Importantly, our identification of NTM in this study dependent on the cohort evaluated. In our study of newly diagnosed cases from multiple outpatient clinics in Addis Ababa, the mycobacteria we isolated consistent exclusively of MTBC for both SNPTB and SPPTB. However, in a second cohort, which consisted of patients referred during follow-up to EPHI from outside, we were able to identify NTM at a prevalence of 7.3% which is similar with a previous study from the same institution (6%) (Mathewos et al., 2015). This prevalence was lower than recent studies reported from St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia of 10.3% (Kahase et al., 2021), a studies from Tanzania, 9.7% (Hoza et al., 2016), and India,8.6% (Umrao et al., 2016). The discordance in the magnitude of isolation from our study may be due to the difference in the study period, population (PTB patient versus PTB suspects) and country versus city. Although, it is important to stress that in these studies, the patient populations under study are likely subject to significant referral bias, collectively they indicate that NTM do contribute to pulmonary infections which can easily be confused with TB. It will be important in future studies to arrive at a more accurate estimate of the prevalence of NTM among newly diagnosed cases of presumptive PTB, in particular SNPTB cases,

The majority of NTM isolates were recovered from presumptive TB patients with an AFB smear negative result. This might be due to the lower sensitivity of AFS for NTM detection or increased sensitivity of fast-growing NTM to the decolourizing reagent used during the procedure (Singhal & Myneedu, 2015). Alternatively, the source of culture grown NTM recovered from smear negative patients in some cases might be from the environment since *M. simiae,* the most frequently isolated species in this study, is primarily found in water (Griffith et al., 2007)

M. avium complex (MAC) has been the most frequently isolated NTM in the majority of studies conducted in both developed and developing countries, including Africa (Gopalaswamy et al., 2020). In contrast, in this study *M. simiae* was the predominant isolate. This is similar to results from Pakistan and Iran (Karamat et al., 2021; Nasiri, Dabiri, et al., 2017). However, our results contrast with other reports from Ethiopia wherein MAC, was most commonly identified in an Adama study (Agonafir et al., 2020), *M. peregrinum* was the commonest in other Ethiopia studies (Kahase et al., 2021) (Workalemahu et al., 2013) and Kenya, *M. fortuitum* the predominant isolate in India (Kaguthi et al., 2019), and MAC the commonest in India (Thangavelu et al., 2021), and Gabon (Epola Dibamba Ndanga et al., 2022).

Unlike MTBC, most NTM are not transmissible via person-to-person means (with the exception of *M. abscessus complex*) and their distribution varies with respect to time, geographical location and the studied population (Honda et al., 2018). For instance, studies from Jimi, Ethiopia and Kenya were conducted on infants, and both found *M. fortuitum* as the most frequent species. On the other hand, *M. simiae* the predominant NTM in this study and reports from Asian countries (Nasiri, Dabiri, et al., 2017) was isolated from presumptive MDR-TB patient placed on second line anti-TB drug treatment highlighting the similarities of *M. simiae* pulmonary infection with PTB in clinical presentation, radiologic findings plus its culture growth characteristics.

M. abscessus complex was the second most frequently isolated NTM species in this study, different from the study reports from Ethiopia (Kahase et al., 2021) and India that observed MAC (Thangavelu et al., 2021), Kenya that found *M. scrofulaceum* (Kaguthi et al., 2019) and Iran that observed *M. fortuitum* (Nasiri, Dabiri, et al., 2017) but similar with a study report from China (Duan et al., 2016). This variation could be due to the difference in the study population between the studies (Duan et

al., 2016). Nevertheless, it is well understood that *M. abscessus* complex is the most frequent rapid growing NTM (80%) isolated from pulmonary samples and associated with clinical diseases (Griffith et al., 2007).

M. fortuitum was the third most frequent isolate in this study, which is different from studies in Adama, Ethiopia (Agonafir et al., 2020), China and Iran (Duan et al., 2016; Nasiri, Dabiri, et al., 2017). Similarly, the above-mentioned factors may contribute for the variation between the study reports. *M. fortuitum* was the predominant NTM isolated from pulmonary samples from infants (Kaguthi et al., 2019; Workalemahu et al., 2013). Identification of this NTM from infants may be of critical clinical importance.

For patients who had clinical and microbiologic information, clinical importance of the identified NTM species was determined based on ATS/IDSA NTM diagnosis criteria. Thus, none of the isolated NTM species identified were clinically important after critical assessment of individual patients. However, many study reports have indicated all of the clinically evaluated NTM species, *M. abscessus* complex, *M. fortuitum* (Wilson et al., 2019), *M. simiae* and *M. szulgai*, have been involved in pulmonary infections particularly in immunocompromised individuals while *M. abscessus* complex *and M. fortuitum* may cause infection irrespective of individuals immune status (Gido et al., 2019; Kurokawa et al., 2020; Nasiri et al., 2018; Victoria et al., 2021).

Unfortunately, the clinical significance of the identified NTM was assessed in only 4 patients out of 35 whereas the remaining 31 patient's medical record was not accessible and/or available. In addition, insufficient data recorded in the available patient records, a problem inherent to retrospective study. The majority of patients

were treated as presumptive MDR-TB, as has been previously described (Cadmus et al., 2016), and compatible with a study report showing similarities in the clinical, radiological and laboratory findings between MDR-TB and NTM pulmonary infections (Kahkouee et al., 2013). Our results show the value of definite diagnosis of presumptive PTB confirmed by laboratory tests including species identification of the isolated NTM.

The WGS based analysis of genetic diversity and drug resistance (DR) detection for PTB (both SNPTB and SPPTB), as well as the comprehensive comparison of clinical, microbiological and genetic features of SNPTB and SPPTB, both of which are the first such studies in Ethiopia could be the strength of the current study. However, he sputum-based sampling which is less sensitive for SNPTB patients and the concentration method used to classify patients may impact the genetic diversity and the magnitude of DR-TB. Inaddition, the clinical importance study on the genotyped NTM from larger cohort of referred patients is also the first such study in Ethiopi will be important in future studies to define the prevalence of NTM infections in a cohort of new presumptive PTB cases, as this may be significantly different,, than that observed in the current studies cohort which was referred after therapy thatmay also have biased the distribution of NTM species in the population.

6. CONCLUSIONS

- The study revealed that a history of prior TB, non-cavitary lesions, normal BMI and increased age are characteristics of SNPTB. There was also a significant discrepancy in classification of SNPTB in different settings reinforcing that the challenge in SNPTB diagnosis.
- The existing MTBC strain distribution among SNPTB appeared to differ from that of SPPTB in the current study.
- In addition, we observed that smear negative MTBC isolates could be further stratified into Xpert positive and negative which magnified the differences in strain distribution, specifically lineage 3 and lineage 4.
- MTBC clustering was detected in a significant number of SNPTB patients, and cases of MDR-TB of SNPTB were found only by culture and not by analysis of fresh sputum.
- The NTM study identified *M. simiae* as the most frequent isolate from pulmonary samples of PTB patients followed by *M. abscessus complex;* however, in a small sample of NTM with adequate clinical records, none were associated with concerning clinical disease.

7. RECOMMENDATIONS

- The finding on the association of history of prior TB and non-cavitary lesions with SNPTB can be used by clinicians for better selection of SNPTB patients for anti-TB drug treatment.
- The discrepancies in classification of SNPTB between facilities laboratories signify the timely revision of the national TB diagnosis guideline that needs further operational research for better recommendation.
- Results from the genetic diversity and drug resistance study warrants yet further improvement of the existing detection methods including GeneXpert.
- Ideally, cost permitting, more widespread more sensitive molecular TB detection tools facilities should be available PTB patients who have smear and Xpert negative tests as soon as possible for better detection of TB and DR-TB to end TB in general.
- Additional prospective studies with larger sample size and more sensitive sample are recommendable for a better understanding of the impact of lineage distribution and DR-TB among SNPTB.
- Further prospective studies with robust genotyping method are recommended to better understand NTM distribution and their clinical significance among PTB patients.

8. REFERENCES

Agonafir, M., Feleke, A., & Belay, G. (2020). Identification of non-tuberculous mycobacteria from clinical specimens referred to Adama TB reference laboratory, Ethiopia. *Ethiopian Journal of Biological Sciences, 19*(2), 145-157.

Akalu G, T., Tessema, B., Sinshaw, W., Amare, M., Diriba, G., Getu, M., Zerihun, B., & Petros, B. (2023). Epidemiology of first-and second-line anti-tuberculosis drug resistance in new pulmonary tuberculosis cases in Addis Ababa metropolitan area, Ethiopia. *Ethiop. J. Health Dev., 37(2) 000-000*, 2023.2004. 2020.23288854.

Akalu, G. T., Tessema, B., & Petros, B. (2022). High proportion of RR-TB and mutations conferring RR outside of the RRDR of the rpoB gene detected in GeneXpert MTB/RIF assay positive pulmonary tuberculosis cases, in Addis Ababa, Ethiopia. *PLoS One, 17*(12), e0277145.

Alemayehu, A., Kebede, A., Neway, S., Tesfaye, E., Zerihun, B., Getu, M., & Petros, B. (2022). A glimpse into the genotype and clinical importance of non tuberculous mycobacteria among pulmonary tuberculosis patients: The case of Ethiopia. *Plos one, 17*(9), e0275159.

Alemayehu, A., Wassie, L., Neway, S., Ayele, S., Assefa, A., Bobosha, K., Petros, B., & Howe, R. (2024). Clinical and Imaging Characteristics of Smear Negative Pulmonary Tuberculosis Patients: A Comparative Study. *Pulmonary Medicine, 2024*(1), 2182088.

Alemu, A., Tadesse, M., Seid, G., Mollalign, H., Eshetu, K., Sinshaw, W., Abebaw, Y., Amare, M., Dagne, B., Diriba, G., Yenew, B., Getu, M., & Zerihun, B. (2020). Does Xpert® MTB/RIF assay give rifampicin resistance results without identified mutation? Review of cases from Addis Ababa, Ethiopia. *BMC Infect Dis, 20*(1), 87.

Aminian, M., Couvin, D., Shabbeer, A., Hadley, K., Vandenberg, S., Rastogi, N., & Bennett, K. P. (2014). Predicting Mycobacterium tuberculosis complex clades using knowledge-based Bayesian networks. *Biomed Res Int, 2014*, 398484.

Andre, E., Goeminne, L., Colmant, A., Beckert, P., Niemann, S., & Delmee, M. (2017). Novel rapid PCR for the detection of Ile491Phe rpoB mutation of Mycobacterium tuberculosis, a rifampicin-resistance-conferring mutation undetected by commercial assays. *Clin Microbiol Infect, 23*(4), 267.e265-267.e267.

Andrews, S. (2010). Babraham bioinformatics – FastQC: a quality control tool for high throughput sequence data. Retrieved from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Anthwal, D., Gupta, R. K., Gomathi, N. S., Tripathy, S. P., Das, D., Pati, S., Panwalkar, N., Desikan, P., Bala, K., Singh, U. B., Bhalla, M., Singhal, R., Verma, A. K., Khayyam, K. U., Myneedu, V. P., Sarin, R., Sharma, S., Bansal, A. K., Gupta, U. D., Patil, S. A., Goyal, A., Gupta, A., Singh, M., Gupta, N. K., Haldar, S., & Tyagi, J. S. (2021). Evaluation of 'TBDetect' sputum microscopy kit for improved detection of Mycobacterium tuberculosis: a multi-centric validation study. *Clin Microbiol Infect, 27*(6), 911.e911-911.e917.

Arora, J., Singhal, R., Bhalla, M., Reza, S., Visalakshi, P., Behera, D., & Myneedu, V. P. (2012). Diagnostic utility of Capilia TB assay for identification of Mycobacterium tuberculosis complex. *Current Research in Tuberculosis, 4*, 13-18.

Barberis, I., Bragazzi, N. L., Galluzzo, L., & Martini, M. (2017). The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. *J Prev Med Hyg, 58*(1), E9-e12.

Barbier, M., & Wirth, T. (2017). The evolutionary history, demography, and spread of the Mycobacterium tuberculosis complex. *Tuberculosis and the Tubercle Bacillus*, 453-473.

Bedewi, Z., Worku, A., Mekonnen, Y., Yimer, G., Medhin, G., Mamo, G., Pieper, R., & Ameni, G. (2017). Molecular typing of Mycobacterium tuberculosis complex isolated from pulmonary tuberculosis patients in central Ethiopia. *BMC Infectious Diseases, 17*(1), 184.

Berhe, D. F., Beyene, G. T., Seyoum, B., Gebre, M., Haile, K., Tsegaye, M., Boltena, M. T., Tesema, E., Kibret, T. C., Biru, M., Siraj, D. S., Shirley, D., Howe,

R., & Abdissa, A. (2021). Prevalence of antimicrobial resistance and its clinical implications in Ethiopia: a systematic review. *Antimicrobial Resistance & Infection Control*, *10*(1), 168.

Blakemore, R., Story, E., Helb, D., Kop, J., Banada, P., Owens, M. R., Chakravorty, S., Jones, M., & Alland, D. (2010). Evaluation of the analytical performance of the Xpert MTB/RIF assay. *Journal of clinical microbiology*, *48*(7), 2495-2501.

Boad-Institute. (2019). Picard tools. Retrieved from http://broadinstitute.github.io/picard/

Boehme, C. C., Nabeta, P., Hillemann, D., Nicol, M. P., Shenai, S., Krapp, F., Allen, J., Tahirli, R., Blakemore, R., Rustomjee, R., Milovic, A., Jones, M., O'Brien, S. M., Persing, D. H., Ruesch-Gerdes, S., Gotuzzo, E., Rodrigues, C., Alland, D., & Perkins, M. D. (2010). Rapid Molecular Detection of Tuberculosis and Rifampin Resistance. *New England Journal of Medicine*, *363*(11), 1005-1015.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *bioinformatics*, *30*(15), 2114-2120.

Boni, F. G., Hamdi, I., Koundi, L. M., Shrestha, K., & Xie, J. (2022). Cytokine storm in tuberculosis and IL-6 involvement. *Infection, Genetics and Evolution*, *97*, 105166.

Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., & Kremer, K. (2002). A new evolutionary scenario for the Mycobacterium tuberculosis complex. *Proceedings of the National Academy of Sciences*, *99*(6), 3684-3689.

Bruchfeld, J., Aderaye, G., Palme, I. B., Bjorvatn, B., Källenius, G., & Lindquist, L. (2000). Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *94*(6), 677-680.

Brudey, K., Driscoll, J. R., Rigouts, L., Prodinger, W. M., Gori, A., Al-Hajoj, S. A., Allix, C., Aristimuño, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P.,

Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J. T., Fauville-Dufaux, M., Ferdinand, S., de Viedma, D. G., Garzelli, C., Gazzola, L., Gomes, H. M., Guttierez, M. C., Hawkey, P. M., van Helden, P. D., Kadival, G. V., Kreiswirth, B. N., Kremer, K., Kubin, M., Kulkarni, S. P., Liens, B., Lillebaek, T., Ly, H. M., Martin, C., Martin, C., Mokrousov, I., Narvskaïa, O., Ngeow, Y. F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M. L., Rüsch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I. G., Singh, U. B., Somoskovi, A., Skuce, R. A., van Soolingen, D., Streicher, E. M., Suffys, P. N., Tortoli, E., Tracevska, T., Vincent, V., Victor, T. C., Warren, R. M., Yap, S. F., Zaman, K., Portaels, F., Rastogi, N., & Sola, C. (2006). Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiology, 6*(1), 23.

Bryant, J. M., Grogono, D. M., Greaves, D., Foweraker, J., Roddick, I., Inns, T., Reacher, M., Haworth, C. S., Curran, M. D., Harris, S. R., Peacock, S. J., Parkhill, J., & Floto, R. A. (2013). Whole-genome sequencing to identify transmission of Mycobacterium abscessus between patients with cystic fibrosis: a retrospective cohort study. *Lancet, 381*(9877), 1551-1560.

Campodónico, V. L., Rifat, D., Chuang, Y.-M., Ioerger, T. R., & Karakousis, P. C. (2018). Altered Mycobacterium tuberculosis Cell Wall Metabolism and Physiology Associated With RpoB Mutation H526D. *Front. Microbiol, 9*, 494.

Campos, L. C., Rocha, M. V., Willers, D. M., & Silva, D. R. (2016). Characteristics of Patients with Smear-Negative Pulmonary Tuberculosis (TB) in a Region with High TB and HIV Prevalence. *PLoS One, 11*(1), e0147933.

Campos, L. C., Rocha, M. V. V., Willers, D. M. C., & Silva, D. R. (2016). Characteristics of Patients with Smear-Negative Pulmonary Tuberculosis (TB) in a Region with High TB and HIV Prevalence. *PLOS ONE, 11*(1), e0147933.

Camprubí, D., Gomila, A., Grijota-Camino, M. D., Soldevila, L., Luque, M. J., Alcaide, F., Dorca, J., & Santin, M. (2020). Infectiousness of patients with smear-negative pulmonary tuberculosis, assessed by Real-time Polymerase Chain Reaction, Xpert®MTB/RIF. *Journal of Infection, 80*(3), 298-300.

Camus, J.-C., Pryor, M. J., Médigue, C., & Cole, S. T. (2002). Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. *Microbiology, 148*(10), 2967-2973.

Cannas, A., Mazzarelli, A., Di Caro, A., Delogu, G., & Girardi, E. (2016). Molecular Typing of Mycobacterium Tuberculosis Strains: A Fundamental Tool for Tuberculosis Control and Elimination. *Infect Dis Rep, 8*(2), 6567.

Carabalí-Isajar, M. L., Rodríguez-Bejarano, O. H., Amado, T., Patarroyo, M. A., Izquierdo, M. A., Lutz, J. R., & Ocampo, M. (2023). Clinical manifestations and immune response to tuberculosis. *World Journal of Microbiology and Biotechnology, 39*(8), 206.

Carey, A. C. (2022). *Disability and the sociological imagination*: SAGE Publications.

Casali, N., Nikolayevskyy, V., Balabanova, Y., Harris, S. R., Ignatyeva, O., Kontsevaya, I., Corander, J., Bryant, J., Parkhill, J., Nejentsev, S., Horstmann, R. D., Brown, T., & Drobniewski, F. (2014). Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nature Genetics, 46*, 279.

Chakravorty, S., Simmons, A. M., Rowneki, M., Parmar, H., Cao, Y., Ryan, J., Banada, P. P., Deshpande, S., Shenai, S., Gall, A., Glass, J., Krieswirth, B., Schumacher, S. G., Nabeta, P., Tukvadze, N., Rodrigues, C., Skrahina, A., Tagliani, E., Cirillo, D. M., Davidow, A., Denkinger, C. M., Persing, D., Kwiatkowski, R., Jones, M., & Alland, D. (2017). The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. *MBio, 8*(4), 12-17.

Chemeda, A., Mihret, A., Abebe, T., Worku, A., & Ameni, G. (2018). Genotyping of mycobacterium tuberculosis isolated from pulmonary tuberculosis patients among people living with HIV in Addis Ababa: Cross-sectional study. *J Clin Tuberc Other Mycobact Dis, 12*, 34-37.

CHEN, Y., HUANG, X., SUN, Q., ZHANG, C., WEN, W., & CHEN, L. (2020). Analysis of drug resistance in patients with smear-negative and culture-

positive pulmonary tuberculosis in Guangdong province in 2016. *Chinese Journal of Antituberculosis, 42*(3), 272-275.

Chihota, V. N., Niehaus, A., Streicher, E. M., Wang, X., Sampson, S. L., Mason, P., Källenius, G., Mfinanga, S. G., Pillay, M., Klopper, M., Kasongo, W., Behr, M. A., Gey van Pittius, N. C., van Helden, P. D., Couvin, D., Rastogi, N., & Warren, R. M. (2018). Geospatial distribution of Mycobacterium tuberculosis genotypes in Africa. *Plos one, 13*(8), e0200632.

Chitale, P., Lemenze, A. D., Fogarty, E. C., Shah, A., Grady, C., Odom-Mabey, A. R., Johnson, W. E., Yang, J. H., Eren, A. M., Brosch, R., Kumar, P., & Alland, D. (2022). A comprehensive update to the Mycobacterium tuberculosis H37Rv reference genome. *Nat Commun, 13*(1), 7068.

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S. J., Lu, X., & Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *fly, 6*(2), 80-92.

Cloete, R., Oppon, E., Murungi, E., Schubert, W.-D., & Christoffels, A. (2016). Resistance related metabolic pathways for drug target identification in Mycobacterium tuberculosis. *BMC Bioinformatics, 17:75*.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., & Barrell, B. G. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature, 393*(6685), 537-544.

Coll, F., McNerney, R., Preston, M. D., Guerra-Assunção, J. A., Warry, A., Hill-Cawthorne, G., Mallard, K., Nair, M., Miranda, A., & Alves, A. (2015). Rapid

determination of anti-tuberculosis drug resistance from whole-genome sequences. *Genome Medicine, 7*, 1-10.

Comín, J., Otal, I., & Samper, S. (2022). In-depth Analysis of IS6110 Genomic Variability in the Mycobacterium tuberculosis Complex. *Front Microbiol, 13*, 767912.

Coscolla, M., Gagneux, S., Menardo, F., Loiseau, C., Ruiz-Rodriguez, P., Borrell, S., Otchere, I. D., Asante-Poku, A., Asare, P., Sánchez-Busó, L., Gehre, F., Sanoussi, C. N., Antonio, M., Affolabi, D., Fyfe, J., Beckert, P., Niemann, S., Alabi, A. S., Grobusch, M. P., Kobbe, R., Parkhill, J., Beisel, C., Fenner, L., Böttger, E. C., Meehan, C. J., Harris, S. R., de Jong, B. C., Yeboah-Manu, D., & Brites, D. (2021). Phylogenomics of Mycobacterium africanum reveals a new lineage and a complex evolutionary history. *Microb Genom, 7*(2).

Couvin, D., David, A., Zozio, T., & Rastogi, N. (2019). Macro-geographical specificities of the prevailing tuberculosis epidemic as seen through SITVIT2, an updated version of the Mycobacterium tuberculosis genotyping database. *Infection, Genetics and Evolution, 72*, 31-43.

Covert, T. C., Rodgers, M. R., Reyes, A. L., & Stelma, G. N., Jr. (1999). Occurrence of nontuberculous mycobacteria in environmental samples. *Applied and environmental microbiology, 65*(6), 2492-2496.

Cozzi, D., Bartolucci, M., Giannelli, F., Cavigli, E., Campolmi, I., Rinaldi, F., & Miele, V. (2024). Parenchymal Cavitations in Pulmonary Tuberculosis: Comparison between Lung Ultrasound, Chest X-ray and Computed Tomography. *Diagnostics, 14*(5), 522.

Damena, D., Tolosa, S., Hailemariam, M., Zewude, A., Worku, A., Mekonnen, B., Mohammed, T., Admasu, A., Chimusa, E. R., Mihret, A., Abebe, T., & Ameni, G. (2019). Genetic diversity and drug susceptibility profiles of Mycobacterium tuberculosis obtained from Saint Peter's TB specialized Hospital, Ethiopia. *PLoS One, 14*(6), e0218545.

Daniel, T. M. (2006). The history of tuberculosis. *Respiratory medicine, 100*(11), 1862-1870.

Davis, J. L., Cattamanchi, A., Cuevas, L. E., Hopewell, P. C., & Steingart, K. R. (2013). Diagnostic accuracy of same-day microscopy versus standard microscopy for pulmonary tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis, 13*(2), 147-154.

de Martino, M., Lodi, L., Galli, L., & Chiappini, E. (2019). Immune Response to Mycobacterium tuberculosis: A Narrative Review. *Frontiers in Pediatrics, 7*.

Dean, A. G. (2010). OpenEpi: open source epidemiologic statistics for public health, version 2.3. 1. *http://www. openepi. com*.

Desta, K., Asrat, D., Lemma, E., Gebeyehu, M., & Feleke, B. (2008). Drug susceptibility of Mycobacterium tuberculosis isolates from smear negative pulmonary tuberculosis patients, Addis Ababa, Ethiopia. *The Ethiopian Journal of Health Development, 22*(2).

Dheda, K., Schwander, S. K., Zhu, B., van Zyl-Smit, R. N., & Zhang, Y. (2010). The immunology of tuberculosis: from bench to bedside. *Respirology, 15*(3), 433-450.

Di Marco, F., Spitaleri, A., Battaglia, S., Batignani, V., Cabibbe, A. M., & Cirillo, D. M. (2023). Advantages of long- and short-reads sequencing for the hybrid investigation of the Mycobacterium tuberculosis genome. *Frontiers in Microbiology, 14*.

Diriba, G., Kebede, A., Tola, H. H., Alemu, A., Yenew, B., Moga, S., Addise, D., Mohammed, Z., Getahun, M., Fantahun, M., Tadesse, M., Dagne, B., Amare, M., Assefa, G., Abera, D., & Desta, K. (2021). Mycobacterial Lineages Associated with Drug Resistance in Patients with Extrapulmonary Tuberculosis in Addis Ababa, Ethiopia. *Tuberculosis Research and Treatment, 2021*, 5239529.

Divangahi, M. (2018). Are tolerance and training required to end TB? *Nature Reviews Immunology, 18*(11), 661-663.

Dookie, N., Rambaran, S., Padayatchi, N., Mahomed, S., & Naidoo, K. (2018). Evolution of drug resistance in Mycobacterium tuberculosis: a review on the molecular determinants of resistance and implications for personalized care. *ANTIMICROB AGENTS CH*, *73*(5), 1138-1151.

Du, D. H., Geskus, R. B., Zhao, Y., Codecasa, L. R., Cirillo, D. M., van Crevel, R., Pascapurnama, D. N., Chaidir, L., Niemann, S., Diel, R., Omar, S. V., Grandjean, L., Rokadiya, S., Ortitz, A. T., Lân, N. H., Hà, Đ. T. M., Smith, E. G., Robinson, E., Dedicoat, M., Nhat, L. T. H., Thwaites, G. E., Van, L. H., Thuong, N. T. T., & Walker, T. M. (2023). The effect of M. tuberculosis lineage on clinical phenotype. *PLOS Global Public Health*, *3*(12), e0001788.

Duan, H., Han, X., Wang, Q., Wang, J., Wang, J., Chu, N., & Huang, H. (2016). Clinical Significance of Nontuberculous Mycobacteria Isolated From Respiratory Specimens in a Chinese Tuberculosis Tertiary Care Center. *Sci Rep*, *6*, 36299.

Eldholm, V., Norheim, G., von der Lippe, B., Kinander, W., Dahle, U. R., Caugant, D. A., Mannsaker, T., Mengshoel, A. T., Dyrhol-Riise, A. M., & Balloux, F. (2014). Evolution of extensively drug-resistant Mycobacterium tuberculosis from a susceptible ancestor in a single patient. *Genome Biol*, *15*(11), 490.

Elkington, P., Polak, M. E., Reichmann, M. T., & Leslie, A. (2022). Understanding the tuberculosis granuloma: the matrix revolutions. *Trends in Molecular Medicine*, *28*(2), 143-154.

Epola Dibamba Ndanga, M., Achimi Agbo Abdul, J. B. P., Edoa, J. R., Chester Mevyann, R., Adegbite, B. R., Mfoumbi, A., Mebiame Biyogho, C., Beh Mba, R., Mahoumbou, J., McCall, M. B. B., Grobusch, M. P., Adegnika, A. A., & Alabi, A. S. (2022). Non-tuberculous mycobacteria isolation from presumptive tuberculosis patients in Lambaréné, Gabon. *Tropical Medicine & International Health*, *27*(4), 438-444.

Esmael, A., Mihret, A., Abebe, T., Mussa, D., Neway, S., Ernst, J., Rengarajan, J., Wassie, L., & Howe, R. (2022). Persistent expression of activation

markers on Mycobacterium tuberculosis-specific CD4 T cells in smear negative TB patients. *Plos one*, *17*(8), e0271234.

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *bioinformatics*, *32*(19), 3047-3048.

Folliero, V., Ferravante, C., Iovane, V., Salvati, A., Crescenzo, L., Perna, R., Corvino, G., Della Rocca, M. T., Panetta, V., Tranfa, A., Greco, G., Baldoni, T., Pagnini, U., Finamore, E., Giurato, G., Nassa, G., Coppola, M., Atripaldi, L., Greco, R., D'Argenio, A., Foti, M. G., Abate, R., Del Giudice, A., Sarnelli, B., Weisz, A., Iovane, G., Pinto, R., Franci, G., & Galdiero, M. (2024). Whole Genome Sequence Dataset of Mycobacterium tuberculosis Strains from Patients of Campania Region. *Sci Data*, *11*(1), 220.

Gaffney, E., Murphy, D., Walsh, A., Connolly, S., Basdeo, S. A., Keane, J., & Phelan, J. J. (2022). Defining the role of neutrophils in the lung during infection: Implications for tuberculosis disease. *Front Immunol*, *13*, 984293.

Gagneux, S. (2018). Ecology and evolution of Mycobacterium tuberculosis. *Nat Rev Microbiol*, *16*(4), 202-213.

Galagan, J. E. (2014). Genomic insights into tuberculosis. *Nature Reviews Genetics*, *15*(5), 307-320.

Garcia-Basteiro, A. L., Ehrlich, J., Bonnet, M., Calnan, M., Graham, S. M., Hermans, S., Jarrett, A., Lewa, R., Mandalakas, A., Martinez, L., Migliori, G. B., Ong, C. W. M., Otero, L., Rangaka, M. X., Raviglione, M. C. B., Seaworth, B., Ssengooba, W., Theron, G., Trajman, A., & Behr, M. A. (2024). A Global Tuberculosis Dictionary: unified terms and definitions for the field of tuberculosis. *The Lancet Global Health*, *12*(5), e737-e739.

Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., & Monsempe, C. (2003). The complete genome sequence of Mycobacterium bovis. *Proceedings of the National Academy of Sciences*, *100*(13), 7877-7882.

Garrison, E., & Marth, G. (2012). Haplotype-based variant detection from short-read sequencing. *arXiv preprint arXiv:1207.3907*.

Getahun, M., Beyene, D., Mollalign, H., Diriba, G., Tesfaye, E., Yenew, B., Taddess, M., Sinshaw, W., & Ameni, G. (2024). Population structure and spatial distribution of Mycobacterium tuberculosis in Ethiopia. *Scientific Reports, 14*(1), 10455.

Gideon, H. P., & Flynn, J. L. (2011). Latent tuberculosis: what the host "sees"? *Immunol Res, 50*(2-3), 202-212.

Gido, R. D. S., Wojciechowski, A. L., & Bajwa, R. P. (2019). Pulmonary infection with Mycobacterium szulgai: A case report. *SAGE open medical case reports, 7*, 2050313X18823448-12050313X18823448.

Goguet de la Salmonière, Y. O., Li, H. M., Torrea, G., Bunschoten, A., van Embden, J., & Gicquel, B. (1997). Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. *J Clin Microbiol, 35*(9), 2210-2214.

Gopalaswamy, R., Shanmugam, S., Mondal, R., & Subbian, S. (2020). Of tuberculosis and non-tuberculous mycobacterial infections–a comparative analysis of epidemiology, diagnosis and treatment. *Journal of biomedical science, 27*(1), 1-17.

Gradmann, C. (2001). Robert Koch and the pressures of scientific research: tuberculosis and tuberculin. *Medical history, 45*(1), 1-32.

Griffith, D. E., Aksamit, T., Brown-Elliott, B. A., Catanzaro, A., Daley, C., Gordin, F., Holland, S. M., Horsburgh, R., Huitt, G., & Iademarco, M. F. (2007). An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med, 175*(4), 367-416.

Grotzke, J. E., & Lewinsohn, D. M. (2005). Role of CD8+ T lymphocytes in control of Mycobacterium tuberculosis infection. *Microbes and Infection, 7*(4), 776-788.

Gupta, R. S., Lo, B., & Son, J. (2018). Phylogenomics and Comparative Genomic Studies Robustly Support Division of the Genus Mycobacterium into an Emended Genus Mycobacterium and Four Novel Genera. *Front Microbiol, 9*, 67.

Gutierrez, M. C., Brisse, S., Brosch, R., Fabre, M., Omaïs, B., Marmiesse, M., Supply, P., & Vincent, V. (2005). Ancient Origin and Gene Mosaicism of the Progenitor of Mycobacterium tuberculosis. *PLoS Pathog 1*(1), e5.

Guyeux, C., Senelle, G., Le Meur, A., Supply, P., Gaudin, C., Phelan, J., Clark, T. G., Rigouts, L., de Jong, B., Sola, C., & Refrégier, G. (2024). Newly Identified Mycobacterium africanum Lineage 10, Central Africa. *Emerg Infect Dis 30*(3), 560.

Hasan, N. A., Davidson, R. M., Epperson, L. E., Kammlade, S. M., Beagle, S., Levin, A. R., de Moura, V. C., Hunkins, J. J., Weakly, N., Sagel, S. D., Martiniano, S. L., Salfinger, M., Daley, C. L., Nick, J. A., & Strong, M. (2021). Population Genomics and Inference of Mycobacterium avium Complex Clusters in Cystic Fibrosis Care Centers, United States. *Emerg Infect Dis*, *27*(11), 2836-2846.

Hayman, J. (1984). Mycobacterium ulcerans: an infection from Jurassic time?

Honda, J. R., Virdi, R., & Chan, E. D. (2018). Global Environmental Nontuberculous Mycobacteria and Their Contemporaneous Man-Made and Natural Niches. *Frontiers in Microbiology*, *9*(2029).

Hoza, A., Mfinanga, S., Rodloff, A., Moser, I., & König, B. (2016). Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: Public health and diagnostic implications for control programmes. *BMC Res Notes 9*, 109.

Hult, C., Mattila, J. T., Gideon, H. P., Linderman, J. J., & Kirschner, D. E. (2021). Neutrophil Dynamics Affect Mycobacterium tuberculosis Granuloma Outcomes and Dissemination. *Frontiers in Immunology, 12*.

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of clinical microbiology*, *26*(11), 2465-2466.

Hunter, R. L., Jagannath, C., & Actor, J. K. (2007). Pathology of postprimary tuberculosis in humans and mice: Contradiction of long-held beliefs. *Tuberculosis, 87*(4), 267-278.

Hussey, M. A., & Zayaitz, A. (2008). Acid-fast stain protocols. *American Society for Microbiology, Washington, DC. USA.*

Imtiaz, S., & Batubara, E. M. (2022). Diagnostic value of bronchoscopy in sputum-negative pulmonary tuberculosis patients and its correlation with clinicoradiological features. *Ann Thorac Med, 17*(2), 124-131.

Ioerger Thomas, R., Feng, Y., Ganesula, K., Chen, X., Dobos Karen, M., Fortune, S., Jacobs William, R., Mizrahi, V., Parish, T., Rubin, E., Sassetti, C., & Sacchettini James, C. (2010). Variation among Genome Sequences of H37Rv Strains of Mycobacterium tuberculosis from Multiple Laboratories. *Journal of Bacteriology, 192*(14), 3645-3653.

Isakova, J., Sovkhozova, N., Vinnikov, D., Goncharova, Z., Talaibekova, E., Aldasheva, N., & Aldashev, A. (2018). Mutations of rpoB, katG, inhA and ahp genes in rifampicin and isoniazid-resistant Mycobacterium tuberculosis in Kyrgyz Republic. *BMC Microbiol, 18*(1), 22.

Islam, M. T., Rabbi, F., Ferdous, S., Parvin, U. S., Hossain, A., & Hossain, M. S. (2014). Evaluation of three immunological tests for the diagnosis of pulmonary tuberculosis in a rural endemic area of Bangladesh. *International Journal of Mycobacteriology, 3*(2), 88-93.

Jagielski, T., van Ingen, J., Rastogi, N., Dziadek, J., Mazur, P. K., & Bielecki, J. (2014). Current Methods in the Molecular Typing of Mycobacterium tuberculosis and Other Mycobacteria. *BioMed Research International, 2014*(1), 645802.

Jaksuwan, R., Tharavichikul, P., Patumanond, J., Chuchottaworn, C., Chanwong, S., Smithtikarn, S., & Settakorn, J. (2017). Genotypic distribution of multidrug-resistant and extensively drug-resistant tuberculosis in northern Thailand. *Infect Drug Resist, 10*, 167-174.

Jennifer L Kelsey, Alice S. Whittemore, Alfred S. Evans, a., & Thompson, W. D. (1996). *Methods in observational epidemiology* (Vol. 10): Oxford University Press.

Jia, X., Yang, L., Dong, M., Chen, S., Lv, L., Cao, D., Fu, J., Yang, T., Zhang, J., Zhang, X., Shang, Y., Wang, G., Sheng, Y., Huang, H., & Chen, F. (2017). The Bioinformatics Analysis of Comparative Genomics of Mycobacterium tuberculosis Complex (MTBC) Provides Insight into Dissimilarities between Intraspecific Groups Differing in Host Association, Virulence, and Epitope Diversity. *Frontiers in Cellular and Infection Microbiology, 7*.

Jones, R. M., Adams, K. N., Eldesouky, H. E., & Sherman, D. R. (2022). The evolving biology of Mycobacterium tuberculosis drug resistance. *Frontiers in Cellular and Infection Microbiology, 12*.

Kaguthi, G., Nduba, V., Murithi, W., & Verver, S. (2019). The Incidence of Non-Tuberculous Mycobacteria in Infants in Kenya. *Journal of Tropical Medicine, 2019*, 1273235.

Kahase, D., Desta, K., Yaregal, Z., Yenew, B., Driba, G., Molalign, H., Solomon, A., & Bekele, F. (2021). Mycobacterium Tuberculosis and Nontuberculous Mycobacteria isolates from presumptive pulmonary tuberculosis patients attending a tertiary hospital in Addis Ababa, Ethiopia. *Ethiopian Journal of Health Sciences, 31*(1).

Kahkouee, S., Esmi, E., Moghadam, A., Karam, M. B., Mosadegh, L., Salek, S., & Tabarsi, P. (2013). Multidrug resistant tuberculosis versus non-tuberculous mycobacterial infections: a CT-scan challenge. *Braz J Infect Dis, 17*(2), 137-142.

Kamerbeek, J., Schouls, L., Kolk, A., Agterveld, M. v., Soolingen, D. v., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., & Embden, J. v. (1997). Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. *Journal of Clinical Microbiology, 35*(4), 907-914.

Karamat, A., Ambreen, A., Ishtiaq, A., Tahseen, S., Rahman, M. A., & Mustafa, T. (2021). Isolation of non-tuberculous mycobacteria among tuberculosis patients, a study from a tertiary care hospital in Lahore, Pakistan. *BMC Infectious Diseases, 21*(1), 381.

Kendall, E. A., Kamoga, C., Kitonsa, P. J., Nalutaaya, A., Salvatore, P. P., Robsky, K., Nakasolya, O., Mukiibi, J., Isooba, D., Cattamanchi, A., Kato-Maeda, M., Katamba, A., & Dowdy, D. W. (2019). Empiric treatment of pulmonary TB in the Xpert era: Correspondence of sputum culture, Xpert MTB/RIF, and clinical diagnoses. *PLOS ONE, 14*(7), e0220251.

Khadka, P., Thapaliya, J., Basnet, R. B., Ghimire, G. R., Amatya, J., & Rijal, B. P. (2019). Diagnosis of tuberculosis from smear-negative presumptive TB cases using Xpert MTB/Rif assay: a cross-sectional study from Nepal. *BMC Infectious Diseases, 19*, 1-7.

Kim, H., & Shin, S. J. (2022). Pathological and protective roles of dendritic cells in Mycobacterium tuberculosis infection: Interaction between host immune responses and pathogen evasion. *Frontiers in Cellular and Infection Microbiology, 12*.

Kim, J. H., Kim, M. J., & Ham, S.-Y. (2019). Clinical characteristics and chest computed tomography findings of smear-positive and smear-negative pulmonary tuberculosis in hospitalized adult patients. *Medicine, 98*(34), e16921.

Kiran, M., Naqvi, S. M. A., Arslan Iqbal, H. B., Butt, A. R. Z., & Shafi, N. (2022). The Diagnostic Performance of Fluorescent Microscopy and MTB/RIF Assay Gene Xpert in Pulmonary Tuberculosis at Tertiary Care Hospital of Lahore. *Pakistan Journal of Medical & Health Sciences, 16*(05), 449-449.

Koch, R. (2010). Die Ätiologie der Tuberkulose. In: Robert Koch-Institut.

Kohlhoff, S. A., & Hammerschlag, M. R. (2015). Treatment of chlamydial infections: 2014 update. *Expert Opinion on Pharmacotherapy, 16*(2), 205-212.

Kurokawa, K., Harada, N., Sasano, H., Takagi, H., Takei, S., Nakamura, A., Kamada, K., Yoshida, A., Kikuchi, K., & Takahashi, K. (2020). Pulmonary infection

due to fluoroquinolone-resistant Mycolicibacterium fortuitum: a case report. *BMC Infectious Diseases, 20*(1), 866.

Lagrange, P. H., Simonney, N., & Herrmann, J. L. (2007). [New immunological tests in the diagnosis of tuberculosis]. *Rev Mal Respir, 24*(4 Pt 1), 453-472.

Lee, Y.-M., Kim, M.-J., & Kim, Y.-J. (2021). Increasing Trend of Nontuberculous Mycobacteria Isolation in a Referral Clinical Laboratory in South Korea. *Medicina, 57*(7), 720.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *bioinformatics, 25*(14), 1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & Subgroup, G. P. D. P. (2009). The sequence alignment/map format and SAMtools. *bioinformatics, 25*(16), 2078-2079.

Li, X., Zhao, W., Han, H., Yang, Z., Bi, F., & He, Y. (2024). Retrospective analysis of the clinical utility of multi-cytokine profiles in smear-negative pulmonary tuberculosis. *Saudi Medical Journal, 45*(7), 658-666.

Lin, C., Russell, C., Soll, B., Chow, D., Bamrah, S., Brostrom, R., Kim, W., Scott, J., & Bankowski, M. J. (2018). Increasing Prevalence of Nontuberculous Mycobacteria in Respiratory Specimens from US-Affiliated Pacific Island Jurisdictions(1). *Emerging infectious diseases, 24*(3), 485-491.

Manderwad, G. P. (2017). Epigenetic Mode of Bacterial Drug Resistance. *Br Biomed Bull, 5*, 295.

Mateyo, K., Kerkhoff, A. D., Dunn, I., Nteeni, M. S., & Muyoyeta, M. (2022). Clinical and radiographic characteristics of presumptive tuberculosis patients previously treated for tuberculosis in Zambia. *Plos one, 17*(1), e0263116.

Mathewos, B., Kebede, N., Kassa, T., Mihret, A., & Getahun, M. (2015). Characterization of mycobacterium isolates from pulmomary tuberculosis suspected cases visiting Tuberculosis Reference Laboratory at Ethiopian Health and Nutrition

Research Institute, Addis Ababa Ethiopia: a cross sectional study. *Asian Pac J Trop Med, 8*(1), 35-40.

Maus, C. E., Plikaytis, B. B., & Shinnick, T. M. (2005). Mutation of tlyA Confers Capreomycin Resistance in *Mycobacterium tuberculosis*. *ANTIMICROB AGENTS CH, 49*, 571-577.

McEvoy, C., Falmer, A., Gey van Pittius, N., Victor, T., Helden, P., & Warren, R. (2007). The role of IS 6110 in the evolution of Mycobacterium tuberculosis. *Tuberculosis, 87*, 393-404.

Mekonnen, D., Munshea, A., Nibret, E., Adnew, B., Herrera-Leon, S., Amor Aramendia, A., Benito, A., Abascal, E., Jacqueline, C., Aseffa, A., & Herrera-Leon, L. (2023). Comparative whole-genome sequence analysis of Mycobacterium tuberculosis isolated from pulmonary tuberculosis and tuberculous lymphadenitis patients in Northwest Ethiopia. *Front Microbiol, 14*, 1211267.

Merid, Y., Hailu, E., Habtamu, G., Tilahun, M., Abebe, M., Hailu, M., Hailu, T., Datiko, D. G., Woldeamanuel, Y., & Aseffa, A. (2021). Molecular Epidemiology of Mycobacterium tuberculosis strains isolated from pulmonary tuberculosis patients in south Ethiopia. *The Journal of Infection in Developing Countries, 15*(09), 1299-1307.

Metlay, J. P., Waterer, G. W., Long, A. C., Anzueto, A., Brozek, J., Crothers, K., Cooley, L. A., Dean, N. C., Fine, M. J., & Flanders, S. A. (2019). Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the American Thoracic Society and Infectious Diseases Society of America. *American journal of respiratory and critical care medicine, 200*(7), e45-e67.

Mihret, A. (2012). The role of dendritic cells in Mycobacterium tuberculosis infection. *Virulence, 3*(7), 654-659.

MoH. (2017). *Guidelines for management of TB, DR-TB and Leprosy in Ethiopia: Sixth Edition*

MoH. (2021). *Guidelines for Clinical and Programmatic Management of TB, TB/HIV, DR-TB and Leprosy in Ethiopia.*

MOH. (2023). *Tuberculosis,Leprosy and Other lung Diseaseas National Strategic Plan: July 2023-June 2030*. Ethiopia: MOH

MOH. (2024). *Annual Tuberculosis and Leprosy Bulletin*. Ethiopia: MOH

Mohammed, H., Oljira, L., Roba, K. T., Ngadaya, E., Ajeme, T., Haile, T., Kidane, A., Manyazewal, T., Fekadu, A., & Yimer, G. (2020). Burden of tuberculosis and challenges related to screening and diagnosis in Ethiopia. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases, 19*, 100158.

Muchsin, S., Priyanto, H., Zulfikar, T., Andayani, N., Yanti, B., & Kurniawan, F. D. (2022). The Difference in Serum Levels of IP-10 in Pulmonary Tuberculosis Patients with Positive AFB and AFB Conversion. *Jurnal Respirologi Indonesia, 42*(3), 199-204.

Mulualem, A., Adey, F., & Gurja, B. (2020). Identification of non-tuberculous mycobacteria from clinical specimens referred to adama tb reference laboratory, ethiopia. *Ethiopian Journal of Biological Sciences, 19*(2), 145–157-145–157.

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B. H., Kumaran, E. A. P., McManigal, B., Achalapong, S., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Babin, F.-X., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Berkley, J. A., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Clotaire Donatien, R., Cook, A. J., Cooper, B., Cressey, T. R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N. P. J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S. J., Duong Bich, T., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garcia, C., Garrett, D., Gastmeier, P., Giref, A. Z., Greer, R. C., Gupta, V., Haller, S., Haselbeck, A., Hay, S. I., Holm, M., Hopkins, S., Hsia, Y., Iregbu, K. C., Jacobs, J., Jarovsky, D., Javanmardi, F., Jenney, A. W. J., Khorana, M.,

Khusuwan, S., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H. H., Lim, C., Lim, K., Limmathurotsakul, D., Loftus, M. J., Lunn, M., Ma, J., Manoharan, A., Marks, F., May, J., Mayxay, M., Mturi, N., Munera-Huertas, T., Musicha, P., Musila, L. A., Mussi-Pinhata, M. M., Naidu, R. N., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C. W., Ochoa, T. J., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Ounchanum, P., Pak, G. D., Paredes, J. L., Peleg, A. Y., Perrone, C., Phe, T., Phommasone, K., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Rattanavong, S., Riddell, A., Roberts, T., Robotham, J. V., Roca, A., Rosenthal, V. D., Rudd, K. E., Russell, N., Sader, H. S., Saengchan, W., Schnall, J., Scott, J. A. G., Seekaew, S., Sharland, M., Shivamallappa, M., Sifuentes-Osornio, J., Simpson, A. J., Steenkeste, N., Stewardson, A. J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Tigoi, C., Turner, C., Turner, P., van Doorn, H. R., Velaphi, S., Vongpradith, A., Vongsouvath, M., Vu, H., Walsh, T., Walson, J. L., Waner, S., Wangrangsimakul, T., Wannapinij, P., Wozniak, T., Young Sharma, T. E. M. W., Yu, K. C., Zheng, P., Sartorius, B., Lopez, A. D., Stergachis, A., Moore, C., Dolecek, C., & Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet, 399*(10325), 629-655.

Naidoo, C. C., & Pillay, M. (2017). Fitness-compensatory mutations facilitate the spread of drug-resistant F15/LAM4/KZN and F28 Mycobacterium tuberculosis strains in KwaZulu-Natal, South Africa. *J Genet, 96*(4), 599-612.

Naluyange, R., Mboowa, G., Komakech, K., Semugenze, D., Kateete, D. P., & Ssengooba, W. (2020). High prevalence of phenotypic pyrazinamide resistance and its association with pncA gene mutations in Mycobacterium tuberculosis isolates from Uganda. *PLOS ONE, 15*(5), e0232543.

Napier, G., Campino, S., Merid, Y., Abebe, M., Woldeamanuel, Y., Aseffa, A., Hibberd, M. L., Phelan, J., & Clark, T. G. (2020). Robust barcoding and identification of Mycobacterium tuberculosis lineages for epidemiological and clinical studies. *Genome Medicine, 12*(1), 114.

Napier, G., Couvin, D., Refrégier, G., Guyeux, C., Meehan, C. J., Sola, C., Campino, S., Phelan, J., & Clark, T. G. (2023). Comparison of in silico predicted

Mycobacterium tuberculosis spoligotypes and lineages from whole genome sequencing data. *Scientific Reports, 13*(1), 11368.

Nasiri, M. J., Dabiri, H., Fooladi, A. A. I., Amini, S., Hamzehloo, G., & Feizabadi, M. M. (2017). High rates of nontuberculous mycobacteria isolation from patients with presumptive tuberculosis in Iran. *New microbes and new infections, 21*, 12-17.

Nasiri, M. J., Haeili, M., Ghazi, M., Goudarzi, H., Pormohammad, A., Imani Fooladi, A. A., & Feizabadi, M. M. (2017). New Insights in to the Intrinsic and Acquired Drug Resistance Mechanisms in Mycobacteria. *Front Microbiol, 8*, 681.

Nasiri, M. J., Heidary, M., Azimi, T., Goudarzi, H., Tabarsi, P., Sarokhalil, D. D., & Feizabadi, M. M. (2018). Mycobacterium simiae pulmonary disease in Iran: systematic review and meta-analysis. *New microbes and new infections, 26*, 118-123.

Ngabonziza, J. C. S., Decroo, T., Maniliho, R., Habimana, Y. M., Van Deun, A., & de Jong, B. C. (2021). Low Cycle Threshold Value in Xpert MTB/RIF Assay May Herald False Detection of Tuberculosis and Rifampicin Resistance: A Study of Two Cases. *Open Forum Infectious Diseases, 8*(2).

Ngabonziza, J. C. S., Loiseau, C., Marceau, M., Jouet, A., Menardo, F., Tzfadia, O., Antoine, R., Niyigena, E. B., Mulders, W., Fissette, K., Diels, M., Gaudin, C., Duthoy, S., Ssengooba, W., André, E., Kaswa, M. K., Habimana, Y. M., Brites, D., Affolabi, D., Mazarati, J. B., de Jong, B. C., Rigouts, L., Gagneux, S., Meehan, C. J., & Supply, P. (2020). A sister lineage of the Mycobacterium tuberculosis complex discovered in the African Great Lakes region. *Nature Communications, 11*(1), 2917.

Nguyen, M.-V. H., Levy, N. S., Ahuja, S. D., Trieu, L., Proops, D. C., & Achkar, J. M. (2019). Factors associated with sputum culture-negative vs culture-positive diagnosis of pulmonary tuberculosis. *JAMA Network Open, 2*(2), e187617-e187617.

Nusrath Unissa, A., & Hanna, L. E. (2017). Molecular mechanisms of action, resistance, detection to the first-line anti tuberculosis drugs: Rifampicin and

pyrazinamide in the post whole genome sequencing era. *Tuberculosis (Edinb), 105*, 96-107.

O'Leary, S., O'Sullivan, M. P., & Keane, J. (2011). IL-10 Blocks Phagosome Maturation in Mycobacterium tuberculosis–Infected Human Macrophages. *American Journal of Respiratory Cell and Molecular Biology, 45*(1), 172-180.

Ocheretina, O., Byrt, E., Mabou, M. M., Royal-Mardi, G., Merveille, Y. M., Rouzier, V., Fitzgerald, D. W., & Pape, J. W. (2016). False-positive rifampin resistant results with Xpert MTB/RIF version 4 assay in clinical samples with a low bacterial load. *Diagn Microbiol Infect Dis, 85*(1), 53-55.

Okoi, C., Anderson, S. T. B., Antonio, M., Mulwa, S. N., Gehre, F., & Adetifa, I. M. O. (2017). Non-tuberculous Mycobacteria isolated from Pulmonary samples in sub-Saharan Africa - A Systematic Review and Meta Analyses. *7*(1), 12002.

Ordaz-Vázquez, A., Torres-González, P., Ferreyra-Reyes, L., Canizales-Quintero, S., Delgado-Sánchez, G., García-García, L., Ponce-De-León, A., Sifuentes-Osornio, J., & Bobadilla-Del-Valle, M. (2023). Mycobacterium tuberculosis lineage 4 associated with cavitations and treatment failure. *BMC Infectious Diseases, 23*(1), 154.

Orgeur, M., Sous, C., Madacki, J., & Brosch, R. (2024). Evolution and emergence of Mycobacterium tuberculosis. *FEMS Microbiology Reviews, 48*(2).

Osei-Wusu, S., Morgan, P., Asare, P., Adams, G., Musah, A. B., Siam, I. M., Gillespie, S. H., Sabiiti, W., & Yeboah-Manu, D. (2021). Bacterial Load Comparison of the Three Main Lineages of Mycobacterium tuberculosis Complex in West Africa. *Frontiers in Microbiology, 12*.

Parte, A. C., Sardà Carbasse, J., Meier-Kolthoff, J. P., Reimer, L. C., & Göker, M. (2020). List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International journal of systematic and evolutionary microbiology, 70*(11), 5607-5612.

Pease, A. S. (1940). Some remarks on the diagnosis and treatment of tuberculosis in antiquity. *Isis, 31*(2), 380-393.

Pethe, K., Swenson, D. L., Alonso, S., Anderson, J., Wang, C., & Russell, D. G. (2004). Isolation of Mycobacterium tuberculosis mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci U S A, 101*(37), 13642-13647.

Phelan, J., de Sessions, P. F., Tientcheu, L., Perdigao, J., Machado, D., Hasan, R., Hasan, Z., Bergval, I. L., Anthony, R., & McNerney, R. (2018). Methylation in Mycobacterium tuberculosis is lineage specific with associated mutations present globally. *Sci Rep, 8*(1), 160.

Phelan, J. E., O'Sullivan, D. M., Machado, D., Ramos, J., Oppong, Y. E. A., Campino, S., O'Grady, J., McNerney, R., Hibberd, M. L., Viveiros, M., Huggett, J. F., & Clark, T. G. (2019). Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. *Genome Medicine, 11*(1), 41.

Prakruthi, S., & Kavitha Sunil, S. (2022). Tuberculosis Diagnosis: Updates and Challenges. In G. Katarzyna & J. Tomas (Eds.), *Bacterial Infectious Diseases Annual Volume 2023* (pp. Ch. 3). Rijeka: IntechOpen.

Queval, C. J., Song, O. R., Carralot, J. P., Saliou, J. M., Bongiovanni, A., Deloison, G., Deboosère, N., Jouny, S., Iantomasi, R., Delorme, V., Debrie, A. S., Park, S. J., Gouveia, J. C., Tomavo, S., Brosch, R., Yoshimura, A., Yeramian, E., & Brodin, P. (2017). Mycobacterium tuberculosis Controls Phagosomal Acidification by Targeting CISH-Mediated Signaling. *Cell Rep, 20*(13), 3188-3198.

Rahlwes, K. C., Dias, B. R. S., Campos, P. C., Alvarez-Arguedas, S., & Shiloh, M. U. (2023). Pathogenicity and virulence of Mycobacterium tuberculosis. *Virulence, 14*(1), 2150449.

Ramasubban, G., Therese, K. L., Lakshmipathy, D., Sridhar, R., Meenakshi, N., & Madhavan, H. N. (2015). Detection of novel and reported mutations in the rpoB, katG and inhA genes in multidrug-resistant tuberculosis isolates: A hospital-based study. *J Glob Antimicrob Resist, 3*(1), 1-4.

Ramon-Luing, L. A., Palacios, Y., Ruiz, A., Téllez-Navarrete, N. A., & Chavez-Galan, L. (2023). Virulence Factors of Mycobacterium tuberculosis as Modulators of Cell Death Mechanisms. *Pathogens, 12*(6).

Ramos-Martinez, A. G., Valtierra-Alvarado, M. A., Garcia-Hernandez, M. H., Hernandez-Pando, R., Castañeda-Delgado, J. E., Cougoule, C., Rivas-Santiago, B., Neyrolles, O., Enciso-Moreno, J. A., Lugo-Villarino, G., & Serrano, C. J. (2019). Variability in the virulence of specific Mycobacterium tuberculosis clinical isolates alters the capacity of human dendritic cells to signal for T cells. *Mem Inst Oswaldo Cruz, 114*, e190102.

Ratnatunga, C. N., Lutzky, V. P., Kupz, A., Doolan, D. L., Reid, D. W., Field, M., Bell, S. C., Thomson, R. M., & Miles, J. J. (2020). The Rise of Non-Tuberculosis Mycobacterial Lung Disease. *Frontiers in Immunology, 11*(303).

Reta, M. A., Tamene, B. A., Abate, B. B., Mensah, E., Maningi, N. E., & Fourie, P. B. (2022). Mycobacterium tuberculosis Drug Resistance in Ethiopia: An Updated Systematic Review and Meta-Analysis. *Trop Med Infect Dis, 7*(10).

Roychowdhury, T., Mandal, S., & Bhattacharya, A. (2015). Analysis of IS6110 insertion sites provide a glimpse into genome evolution of Mycobacterium tuberculosis. *Scientific reports, 5*, 12567.

Sakatos, A., Babunovic, G. H., Chase, M. R., Dills, A., Leszyk, J., Rosebrock, T., Bryson, B., & Fortune, S. M. (2018). Posttranslational modification of a histone-like protein regulates phenotypic resistance to isoniazid in mycobacteria. . *Sci Adv, 4.*, eaao1478.

Salvato, R., Schiefelbein, S., Barcellos, R., Praetzel, B., Anusca, I., Esteves, L., Halon, M., Unis, G., Dias, C., & Miranda, S. (2019). Molecular characterisation of multidrug-resistant Mycobacterium tuberculosis isolates from a high-burden tuberculosis state in Brazil. *Epidemiology & Infection, 147*, e216.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A, 74*(12), 5463-5467.

Sankar, P., & Mishra, B. B. (2023). Early innate cell interactions with Mycobacterium tuberculosis in protection and pathology of tuberculosis. *Front Immunol, 14*, 1260859.

Satam, H., Joshi, K., Mangrolia, U., Waghoo, S., Zaidi, G., Rawool, S., Thakare, R. P., Banday, S., Mishra, A. K., Das, G., & Malonia, S. K. (2023). Next-Generation Sequencing Technology: Current Trends and Advancements. *Biology (Basel), 12*(7).

Schiff, H. F., Jones, S., Achaiah, A., Pereira, A., Stait, G., & Green, B. (2019). Clinical relevance of non-tuberculous mycobacteria isolated from respiratory specimens: seven year experience in a UK hospital. *Scientific reports, 9*(1), 1730-1730.

Shabbeer, A., Cowan, L. S., Ozcaglar, C., Rastogi, N., Vandenberg, S. L., Yener, B., & Bennett, K. P. (2012). TB-Lineage: an online tool for classification and analysis of strains of Mycobacterium tuberculosis complex. *Infect Genet Evol, 12*(4), 789-797.

Shell, S. S., Prestwich, E. G., Baek, S.-H., Shah, R. R., Sassetti, C. M., Dedon, P. C., & Fortune, S. M. (2013). DNA Methylation Impacts Gene Expression and Ensures Hypoxic Survival of *Mycobacterium tuberculosis*. *PLOS Pathogens, 9*, 9

Shitikov, E., & Bespiatykh, D. (2023). A revised SNP-based barcoding scheme for typing Mycobacterium tuberculosis complex isolates. *mSphere, 8*(4), e00169-00123.

Shuaib, Y. A., Utpatel, C., Kohl, T. A., Barilar, I., Diricks, M., Ashraf, N., Wieler, L. H., Kerubo, G., Mesfin, E. A., Diallo, A. B., Al-Hajoj, S., Ndung'u, P., Fitzgibbon, M. M., Vaziri, F., Sintchenko, V., Martinez, E., Viegas, S. O., Zhou, Y., Azmy, A., Al-Amry, K., Godreuil, S., Varma-Basil, M., Narang, A., Ali, S., Beckert, P., Dreyer, V., Kabwe, M., Bates, M., Hoelscher, M., Rachow, A., Gori, A., Tekwu, E. M., Sidze, L. K., Jean-Paul, A. A., Beng, V. P., Ntoumi, F., Frank, M., Diallo, A. G., Mboup, S., Tessema, B., Beyene, D., Khan, S. N., Diel, R., Supply, P., Maurer, F.

P., Hoffmann, H., Niemann, S., & Merker, M. (2022). Origin and Global Expansion of Mycobacterium tuberculosis Complex Lineage 3. *Genes, 13*(6), 990.

Siddiqi, S. H., & Rüsch-Gerdes, S. (2006). *MGIT procedure manual. Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland*.

Silva, D. R., Muñoz-Torrico, M., Duarte, R., Galvão, T., Bonini, E. H., Arbex, F. F., Arbex, M. A., Augusto, V. M., Rabahi, M. F., & Mello, F. C. Q. (2018). Risk factors for tuberculosis: diabetes, smoking, alcohol use, and the use of other drugs. *J Bras Pneumol, 44*(2), 145-152.

Silva, M. L., Cá, B., Osório, N. S., Rodrigues, P. N., Maceiras, A. R., & Saraiva, M. (2022). Tuberculosis caused by Mycobacterium africanum: Knowns and unknowns. *PLoS pathogens, 18*(5), e1010490.

Simieneh, A., Tadesse, M., Kebede, W., Gashaw, M., & Abebe, G. (2022). Combination of Xpert® MTB/RIF and DetermineTM TB-LAM Ag improves the diagnosis of extrapulmonary tuberculosis at Jimma University Medical Center, Oromia, Ethiopia. *PLOS ONE, 17*(2), e0263172.

Singhal, R., & Myneedu, V. P. (2015). Microscopy as a diagnostic tool in pulmonary tuberculosis. *International Journal of Mycobacteriology, 4*(1), 1-6.

Sinshaw, W., Kebede, A., Bitew, A., Tesfaye, E., Tadesse, M., Mehamed, Z., Yenew, B., Amare, M., Dagne, B., Diriba, G., Alemu, A., Getahun, M., Fikadu, D., Desta, K., & Tola, H. H. (2019). Prevalence of tuberculosis, multidrug resistant tuberculosis and associated risk factors among smear negative presumptive pulmonary tuberculosis patients in Addis Ababa, Ethiopia. *BMC Infectious Diseases, 19*(1), 641.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics, 22*(21), 2688-2690.

Steingart, K. R., Schiller, I., Horne, D. J., Pai, M., Boehme, C. C., & Dendukuri, N. (2014). Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev, 2014*(1), Cd009593.

Stucki, D., Malla, B., Hostettler, S., Huna, T., Feldmann, J., Yeboah-Manu, D., Borrell, S., Fenner, L., Comas, I., Coscollà, M., & Gagneux, S. (2012). Two New Rapid SNP-Typing Methods for Classifying Mycobacterium tuberculosis Complex into the Main Phylogenetic Lineages. *PLOS ONE, 7*(7), e41253.

Supply, P., Marceau, M., Mangenot, S., Roche, D., Rouanet, C., Khanna, V., Majlessi, L., Criscuolo, A., Tap, J., & Pawlik, A. (2013). Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of Mycobacterium tuberculosis. *Nat Genet, 45*(2), 172-179.

Swanepoel, C. C., & Loots, D. T. (2014). The use of functional genomics in conjunction with metabolomics for Mycobacterium tuberculosis research. *Disease markers, 2014*(1), 124218.

Tadesse, M., Aragaw, D., Rigouts, L., & Abebe, G. (2016). Increased detection of smear-negative pulmonary tuberculosis by GeneXpert MTB/RIF® assay after bleach concentration. *International Journal of Mycobacteriology, 5*(2), 211-218.

Tessema, B., Beer, J., Merker, M., Emmrich, F., Sack, U., Rodloff, A. C., & Niemann, S. (2013). Molecular epidemiology and transmission dynamics of Mycobacterium tuberculosis in Northwest Ethiopia: new phylogenetic lineages found in Northwest Ethiopia. *BMC Infectious Diseases, 13*(1), 131.

Thangavelu, K., Krishnakumariamma, K., Pallam, G., Prakash, D. D., Chandrashekar, L., Kalaiarasan, E., Das, S., Muthuraj, M., & Joseph, N. M. (2021). Prevalence and speciation of non-tuberculous mycobacteria among pulmonary and extrapulmonary tuberculosis suspects in South India. *Journal of Infection and Public Health, 14*(3), 320-323.

Tiamiyu, A. B., Iliyasu, G., Dayyab, F. M., Habib, Z. G., Tambuwal, S. H., Animashaun, A. O., Galadanci, H., Bwala, S. A., Lawson, L., & Habib, A. G. (2020). A descriptive study of smear negative pulmonary tuberculosis in a high HIV burden patient's population in North Central Nigeria. *PLOS ONE, 15*(9), e0238007.

Tilahun, M., Ameni, G., Desta, K., Zewude, A., Yamuah, L., Abebe, M., & Aseffa, A. (2018). Molecular epidemiology and drug sensitivity pattern of

Mycobacterium tuberculosis strains isolated from pulmonary tuberculosis patients in and around Ambo Town, Central Ethiopia. *PloS one, 13*(2), e0193083.

Torokaa, P. R., Majigo, M. V., Kileo, H., Urio, L., Mbwana, M. R., Monah, M. C., Ntibabara, S. S., Kimambo, J., Seleman, P., Franklin, C., Balama, R., Kisonga, R. M., & Joachim, A. (2024). The pattern of rpoB gene mutation of Mycobacterium tuberculosis and predictors of rifampicin resistance detected by GeneXpert MTB/RIF assay in Tanzania. *PLOS ONE, 19*(8), e0296563.

Uddin, M. K., Chowdhury, M. R., Ahmed, S., Rahman, M. T., Khatun, R., van Leth, F., & Banu, S. (2013). Comparison of direct versus concentrated smear microscopy in detection of pulmonary tuberculosis. *BMC Res Notes, 6*, 291.

Umrao, J., Singh, D., Zia, A., Saxena, S., Sarsaiya, S., Singh, S., Khatoon, J., & Dhole, T. N. (2016). Prevalence and species spectrum of both pulmonary and extrapulmonary nontuberculous mycobacteria isolates at a tertiary care center. *International Journal of Mycobacteriology, 5*(3), 288-293.

Urbanowski, M. E., Ordonez, A. A., Ruiz-Bedoya, C. A., Jain, S. K., & Bishai, W. R. (2020). Cavitary tuberculosis: the gateway of disease transmission. *The Lancet Infectious Diseases, 20*(6), e117-e128.

Van Soolingen, D., Hermans, P., De Haas, P., Soll, D., & Van Embden, J. (1991). Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *Journal of Clinical Microbiology, 29*(11), 2578-2586.

Victoria, L., Gupta, A., Gómez, J. L., & Robledo, J. (2021). Mycobacterium abscessus complex: A Review of Recent Developments in an Emerging Pathogen. *Frontiers in Cellular and Infection Microbiology, 11*(338).

Warren, E., Teskey, G., & Venketaraman, V. (2017). Effector Mechanisms of Neutrophils within the Innate Immune System in Response to Mycobacterium tuberculosis Infection. *J Clin Med, 6*(2).

Warren, R., Streicher, E., Sampson, S., Van Der Spuy, G., Richardson, M., Nguyen, D., Behr, M., Victor, T., & Van Helden, P. (2002). Microevolution of the direct repeat region of Mycobacterium tuberculosis: implications for interpretation of spoligotyping data. *Journal of Clinical Microbiology*, *40*(12), 4457-4465.

WHO. (2012). *Tuberculosis laboratory biosafety manual*: World Health Organization.

WHO. (2015). *End TB Strategy (2016-2035). World Health Organization.*

WHO. (2019). WHO consolidated guidelines on drug-resistant tuberculosis treatment. In: World Health Organization.

WHO. (2022a). *Global tuberculosis report 2022. Geneva Swizerland : World Health Organization 2022* (9240029435).

WHO. (2022b). *WHO consolidated guidelines on tuberculosis. Module 4: treatment-drug-resistant tuberculosis treatment, 2022 update*: World Health Organization.

WHO. (2022c). *WHO consolidated guidelines on tuberculosis. Module 4: treatment-drug-susceptible tuberculosis treatment*: World Health Organization.

WHO. (2023a). Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance.World Health Organization (WHO). In (2nd ed.). Geneva: World Health Organization.

WHO. (2024). *WHO consolidated guidelines on tuberculosis. Module 3: diagnosis–rapid diagnostics for tuberculosis detection*: World Health Organization.

WHO. (2023b). *WHO's global tuberculosis report: World Health Organization.*

Wiens, K. E., Woyczynski, L. P., Ledesma, J. R., Ross, J. M., Zenteno-Cuevas, R., Goodridge, A., Ullah, I., Mathema, B., Djoba Siawaya, J. F., & Biehl, M. H. (2018). Global variation in bacterial strains that cause tuberculosis disease: a systematic review and meta-analysis. *BMC medicine*, *16*, 1-13.

Wilson, J. W., Jagtiani, A. C., & Wengenack, N. L. (2019). Mycobacterium scrofulaceum disease: experience from a tertiary medical centre and review of the literature. *Infect Dis (Lond), 51*(8), 602-609.

Wolf, A. J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K., & Ernst, J. D. (2008). Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J Exp Med, 205*(1), 105-115.

Wondale, B., Keehwan, K., Medhin, G., Teklu, T., Mohammed, T., Tolosa, S., Zewude, A., Amsalu, F., Pieper, R., & Ameni, G. (2020). Molecular epidemiology of clinical Mycobacterium tuberculosis complex isolates in South Omo, Southern Ethiopia. *BMC Infectious Diseases, 20*, 1-12.

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome biology, 20*(1), 257.

Workalemahu, B., Berg, S., Tsegaye, W., Abdissa, A., Girma, T., Abebe, M., & Aseffa, A. (2013). Genotype diversity of Mycobacterium isolates from children in Jimma, Ethiopia. *BMC Research Notes, 6*(1), 352.

Worku, G., Gumi, B., Mohammedbirhan, B., Girma, M., Sileshi, H., Hailu, M., Wondimu, A., Ashagre, W., Tschopp, R., Carruth, L., & Ameni, G. (2022). Molecular epidemiology of tuberculosis in the Somali region, eastern Ethiopia. *Frontiers in Medicine, 9*.

Xia, Q., Zhao, L.-l., Li, F., Fan, Y.-m., Chen, Y.-y., Wu, B.-b., Liu, Z.-w., Pan, A.-z., & Zhu, M. (2015). Phenotypic and Genotypic Characterization of Pyrazinamide Resistance among Multidrug-Resistant Mycobacterium tuberculosis Isolates in Zhejiang, China. *Antimicrobial Agents and Chemotherapy, 59*(3), 1690-1695.

Yang, X., & Feng, H. (2023). Changes of lymphocyte subsets in smear-negative pulmonary tuberculosis. *Indian Journal of Pathology and Microbiology, 66*(2).

Yenew, B., Kebede, A., Alemu, A., Diriba, G., Mehammed, Z., Amare, M., Dagne, B., Sinshaw, W., Tesfaye, E., Beyene, D., & Abegaz, W. E. (2024). Genotypic and phenotypic drug resistance patterns of Mycobacterium tuberculosis isolated from presumptive pulmonary tuberculosis patients in Ethiopia: A multicenter study. *PLOS ONE, 19*(5), e0303460.

Yimer, S. A., Kalayou, S., Homberset, H., Birhanu, A. G., Riaz, T., Zegeye, E. D., Lutter, T., Abebe, M., Holm-Hansen, C., Aseffa, A., & Tønjum, T. (2020). Lineage-Specific Proteomic Signatures in the Mycobacterium tuberculosis Complex Reveal Differential Abundance of Proteins Involved in Virulence, DNA Repair, CRISPR-Cas, Bioenergetics and Lipid Metabolism. *Frontiers in Microbiology, 11*.

Yun, Y. J., Lee, J. S., Yoo, J. C., Cho, E., Park, D., Kook, Y. H., & Lee, K. H. (2018). Patterns of rpoC Mutations in Drug-Resistant Mycobacterium tuberculosis Isolated from Patients in South Korea. *Tuberc Respir Dis (Seoul)*, 1738-3536.

Zhou, Y., van den Hof, S., Wang, S., Pang, Y., Zhao, B., Xia, H., Anthony, R., Ou, X., Li, Q., Zheng, Y., Song, Y., Zhao, Y., & van Soolingen, D. (2017). Association between genotype and drug resistance profiles of Mycobacterium tuberculosis strains circulating in China in a national drug resistance survey. *PLOS ONE, 12*(3), e0174197.

APPENDIX

Appendix 1. Sample size calculation

Sample Size for Unmatched Case-Control Study

For:

Two-sided confidence level(1-alpha)	95
Power (% chance of detecting)	80
Ratio of Controls to Cases	1
Hypothetical proportion of controls with exposure	71
Hypothetical proportion of cases with exposure:	54
Least extreme Odds Ratio to be detected:	0.48

	Kelsey	**Fleiss**	**Fleiss with CC**
Sample Size – Cases	128	127	139
Sample Size – Controls	128	127	139
Total sample size:	256	254	278

CC = continuity correction
Results are rounded up to the nearest integer.

Appendix 2. Supplementary Table 1. Spoligotypes of 116 MTBC isolates, Addis Ababa, Ethiopia,2021.

SPPTB					SNPTB			
Ser. o	Octal	SIT	Family	Lineage name	Octal	SIT	Family	Lineage name
	703777740003771	Orphan	CAS1-DELHI	EAI	377777777760771	7	T	EAI
	703777740003771	26	CAS1-DELHI	EAI	703377400001771	21	CAS1-KILL	EAI
	703777740002171	26	CAS1-DELHI	EAI	703377400001771	21	CAS1-KILL	EAI
	703777700003771	26	CAS1-DELHI	EAI	703377400001771	21	CAS1-KILL	EAI
	777737777760771	37	T3	EA	703377400001771	21	CAS1-KILL	EAI
	777737777760771	37	T3	EA	703777740003171	26	CAS1-DELHI	EAI
	777777404760771	41	TUR	EA	703777740003171	26	CAS1-DELHI	EAI
	777777404760771	41	TUR	EA	703777700003771	26	CAS1-DELHI	EAI
	777777404760771	41	TUR	EA	777737777760771	37	T3	EA
	777777607760771	42	LAM9	EA	777777607760771	42	LAM9	EA
	777777777760731	52	T2	EA	777777777720771	50	H3	EA
	777777777760731	52	T2	EA	777777777760771	53	T	EA
	777777777760771	53	T	EA	777777777760771	53	T	EA
	777777777760771	53	T	EA	777777777760771	53	T	EA

777777777760771	53	T	EA	777777777760771	53	T	EA
777777777760771	53	T	EA	777777777760771	53	T	EA
777777777760771	53	T	EA	777777777763771	54	UNKNOWN	EA
777777777760771	53	T	EA	777777777763771	54	MANU2	EA
777777777760771	53	T	EA	777777777720631	134	H3	EA
777777777760771	53	T	EA	777000377760771	149	T3_ETH	EA
777777777763771	54	MANU2	EA	777000377760771	149	T3_ETH	EA
777777777763771	54	MANU2	EA	777000377760771	149	T3_ETH	EA
777776777760771	119	X1	EA	777000377760771	149	T3_ETH	EA
777777775720771	121	H3	EA	777737777760601	158	T3	EA
777776777760601	137	X2	EA	777777777760671	245	T	EA
777000377760771	149	T3_ETH	EA	774777777420771	262	H3-URAL-1	EA
777000377760771	149	T3_ETH	EA	777776777760731	336	X1	EA
777000377760771	149	T3_ETH	EA	700000007177771	910	AFR	MA
777000377760771	149	T3_ETH	EA	777777774000000	1353	H1	EA
777000377760771	149	T3_ETH	EA	777777403760771	1688	LAM (T)	EA
777000377760771	149	T3_ETH	EA	777777403760771	1689	LAM	EA

777000377760771	149	T3_ETH	EA	777347777760771	1821	T	EA
777000377760771	149	T3_ETH	EA	703777700003171	2392	CAS1-DELIH	EAI
777000377760771	149	T3_ETH	EA	000017777760771	New	T3-OSA	EA
777737777760601	158	T3	EA	513767777603771	New	MANU2	IO
737777777760771	205	T	EA	713377400001771	New	CAS1-KILL	EAI
777777603760771	230	LAM	EA	713777740003171	New	CAS1-DELHI	EAI
777775777760731	554	T2	EA	713777740003171	New	CAS1-DELHI	EAI
777757777720771	764	H3	EA	003777777761171	New	MANU2	EA
777757777720771	764	H3	EA	627737740003171	New	CAS1-DELHI	IO
777777717760771	771	T	EA	773757777763671	New	MANU2	EA
777777777420771	777	H3-URAL1	EA	777375777760771	Orphan	T	EA
777777777420771	777	H3-URAL1	EA				
700000007177771	910	AFR	MA				
777737777420731	1134	H3-URAL-1	EA				
703777600003771	1093	CAS1-DELIH	EAI				
703673740003171	New	CAS1-DELIH	EAI				
777377777760771	1166	T	EA				

	777777403760771	1690	T	EA
	703777700003171	2392	CAS1-DELIH	EAI
	777737757760771	2409	T3	EA
	777777777763431	New	MANU2	EA
	571046177763771	New	MANU2	EA
	303777740003571	New	CAS1-DELHI	EAI
	777775777762771	New	MANU2	EA
	400004757760771	New	T1-RUS2	EA
	777767677760660	New	T	EA
	403200077760771	New	T3-ETH	EA
	511047377760771	New	T3-OSA	EA
	777737777763731	New	MANU2	EA
	773775777760731	New	T2	EA
	411703777760671	New	T3-OSA	EA
	476737777760771	New	T3	EA
	777000377761771	New	T3_ETH	EA
	777342377760771	New	T	EA

	777740370000000	New	ZERO	EA
	474137777760771	New	S	EA
	713777747763771	New	MANU2	EA
	777377677420771	New	H3-URAL1	EA
	777737477760771	Orphan	H37RV(T)	EA
	701777740003171	Orphan	CAS1-DELHI	EAI
	777777703760771	Orphan	CAMEROON	EA
	777000177760771	Orphan	T3-ETH	EA
	777777740003171	Orphan	EAI7-BDG2	IO

EA: Euro American, EAI East African Indian, And IO: Indo Oceanic MA; *M. africanum*, SNPTB: smear negative PTB, SPPTB: smear positive PTB

Appendix 3. Supplementary Table 2. Spoligotyping and whole genome sequencing data of 44 PTB patients, Addis Ababa, Ethiopia, 2021.

	WGS lineage	**Spoligotyping**			**PTB type**
AHRI No		SIT	family/ clade	Lineage name	
1.	Lineage 4.2.2.2	53	T	EA	SPPTB
2.	Lineage 3	26	CAS1-DELHI	EAI	SPPTB
3.	Lineage 4.2.1	53	T	EA	SNPTB
4.	Lineage 4.2.2.1	42	LAM7-TUR(Turkey)	EA	SPPTB
5.	Lineage 4.2.2.2	53	T	EA	SPPTB
6.	Lineage 4.1.2	53	T	EA	SNPTB
7.	Lineage 3.1.1	ORPHAN	CAS1-DELHI	EAI	SPPTB
8.	Lineage 4.2.2.2	134	H3	EA	SNPTB
9.	Lineage 4.3.1	ORPHAN	Cameroon	EA	SPPTB
10.	Lineage 3	2392	CAS1-DELHI	EAI	SNPTB
11.	Lineage 4.8	53	T	EA	SNPTB
12.	Lineage 4.2.2.2	149	T3_ETH	EA	SNPTB
13.	Lineage 4.6.3	53	T	EA	SPPTB
14.	Lineage4.8	262	H3-Ural-1	EA	SPPTB
15.	lineage4.6	554	T2	EA	SPPTB
16.	lineage 4.2.2.2	149	T3_ETH	EA	SNPTB
17.	lineage3	NEW	CAS1-DELHI	EAI	SNPTB
18.	lineage 4.1.2.1	50	H3	EA	SNPTB
19.	lineage 4.2.2.2	336	X1	EA	SNPTB
20.	lineage 3.1.1	21	CAS1-KILL	EAI	SNPTB
21.	lineage 4	7	T	EA	SNPTB
22.	lineage 7	910	AFR	MA	SNPTB
23.	lineage3	NEW	CAS1-DELHI	EAI	SNPTB
24.	lineage4.2.2.2	119	X1	EA	SPPTB
25.	lineage4.2.2.2	149	T3_ETH	EA	SPPTB
26.	lineage 4.2.2.2	149	T3_ETH	EA	SNPTB

27.	lineage4.1.2.1	54	MANU2	EA	SPPTB
28.	lineage4.1.1.3	NEW	MANU2	EA	SPPTB
29.	lineage4.6	NEW	T1-RUS2	EA	SPPTB
30.	lineage 4.1.2.1	121	H3	EA	SPPTB
31.	lineage4.2.1	1134	H3-Ural-1	EA	SPPTB
32.	lineage3	NEW	MANU2	EA	SPPTB
33.	lineage4.2.2.2	52	T2	EA	SPPTB
34.	lineage4.3.4.1	771	T	EA	SPPTB
35.	lineage3	NEW	CAS1-DELHI	EAI	SPPTB
36.	lineage4.1.2	ORPHAN	H37Rv(T)	EA	SPPTB
37.	lineage 3.1.1	21	CAS1-KILL	EAI	SNPTB
38.	lineage4.6.3	2409	AMBIGIOUS; T3, T5	EA	SPPTB
39.	lineage4.2.2.2	149	T3_ETH	EA	SPPTB
40.	lineage4.2.2.2	149	T3_ETH	EA	SPPTB
41.	lineage4.8	NEW	T	EA	SPPTB
42.	lineage4.2.1	53	T	EA	SPPTB
43.	lineage 3	NEW	MANU2	EA	SPPTB
44.	lineage4.2.2.2	52	T2	EA	SPPTB

EA: Euro American, EAI East African Indian, MA; *M. africanum*

Appendix 4. Supplementary Table 3. Clinical and laboratory data of patients that NTM was isolated from their specimens

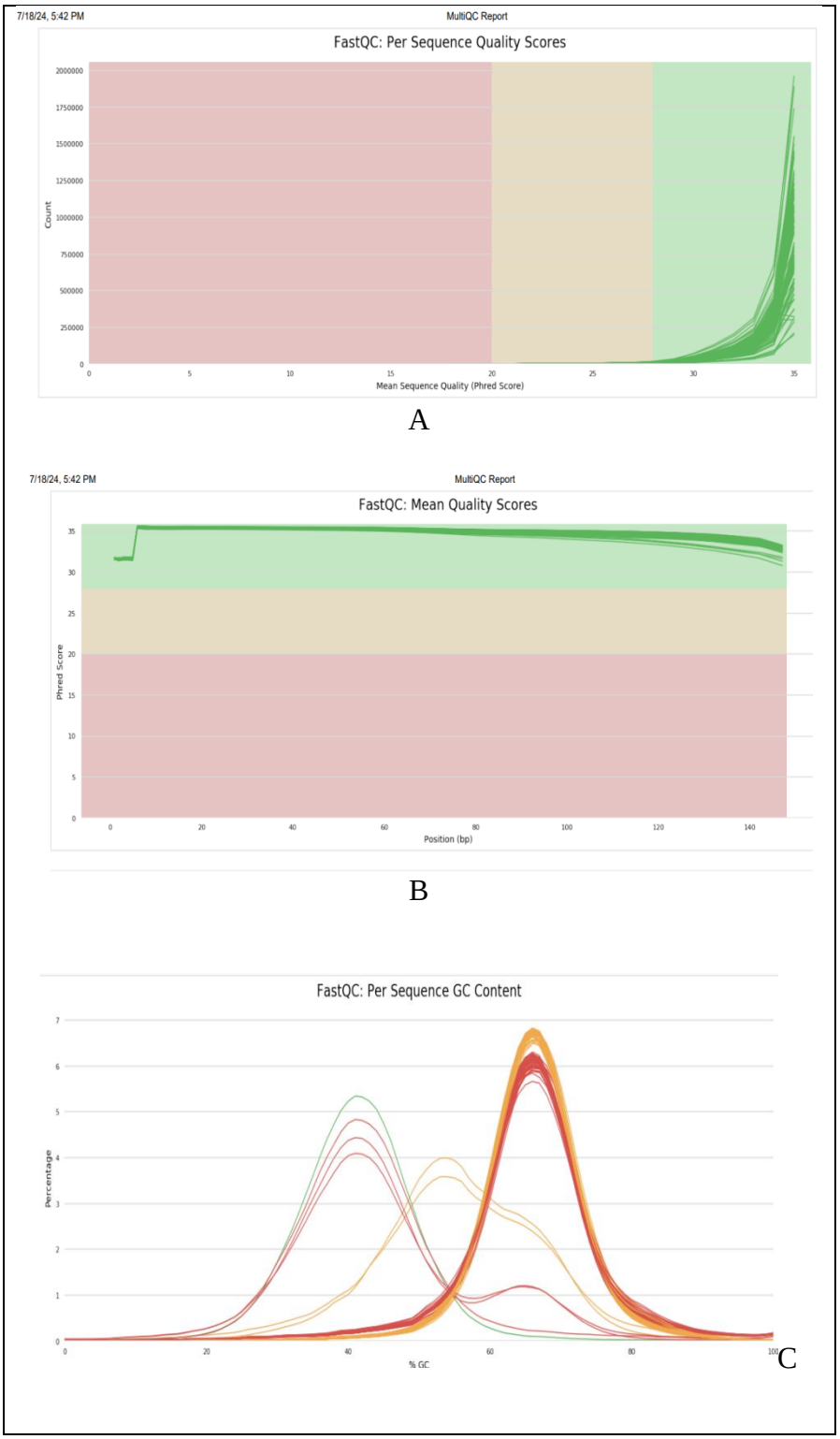
Serial No	Age	Sex	HIV Status	TB classification	Previous Treatment	Reason for test	Follow-up Month	Specimen Type	AFB Smear Result	Culture Result
1.	40	Female	Non-Reactive	New case	NR	Follow up	3	Sputum	Negative	NTM
2.	20	Female	NR	NR	First line	Follow up	5	Sputum	Negative	NTM
3.	38	Female	Reactive	NR	New	Follow up	15	Sputum	Negative	NTM
4.	36	Female	Unknown	Relapse case	NR	NR	NR	Sputum	Negative	NTM
5.	30	Male	NR	NR	First line	Diagnosis	NR	Sputum	Negative	NTM
6.	35	Female	Reactive	NR	NR	Follow up	16	Sputum	Negative	NTM
7.	84	Male	NR	New case	NR	Follow up	2	Sputum	Scanty	NTM
8.	18	Female	Non-Reactive	New case	second line	Follow up	15	Sputum	Negative	NTM
9.	25	Female	Unknown	Relapse case	First line	Follow up	15	Sputum	Negative	NTM
10.	20	Female	NR	Treatment failure	second line	Follow up	7	Sputum	Negative	NTM
11.	18	Female	NR	New case		Follow up	5	Sputum	Negative	NTM
12.	14	Male	Reactive	Treatment failure	second line	Follow up	2	Sputum	Positive +2	NTM
13.	25	Female	Non-Reactive	NR	NR	Follow up	12	Sputum	Negative	NTM
14.	28	Male	NR	Treatment failure	First line	Follow up	0	Sputum	Positive +2	NTM
15.		Male	NR	New case	3=second line	Follow up	5	Sputum	Negative	NTM
16.	9	Female	NR		second line	Follow up	15	Sputum	Negative	NTM
17.	22	Male	Non-Reactive	New case	First line	Follow up	0	Sputum	Negative	NTM
18.	54	Male	Non-Reactive		First line		NR	Sputum	Positive +3	NTM
19.	55	Male	Non-Reactive	Treatment failure	New	Follow up	14	Sputum	Positive +2	NTM
20.	55	Male	NR	New case	First line	NR	6	Sputum	Positive +3	NTM
21.	55	Male	Unknown		NR	NR	NR	Sputum	Positive +1	NTM
22.		Female	Non-Reactive	New case	First line	Follow up	3	Sputum	Negative	NTM
23.	30	Male	Unknown	Relapse case	second line	Diagnosis	NR	Sputum	Negative	NTM
24.	22	Female	Non-Reactive			Follow up	5	Sputum	Negative	NTM
25.	22	Female	NR	NR	First line	Follow up	7	Sputum	Negative	NTM
26.	28	Male	Reactive	New case		Follow up	2	Sputum	Negative	NTM

27.	26	Female	Reactive	Treatment failure	First line	Follow up	13	Sputum	Negative	NTM
28.	25	Male	NR	NR	NR	Follow up	17	Sputum	Negative	NTM
29.	30	Female	Reactive	Relapse case	second line	Follow up	22	Sputum	Negative	NTM
30.	21	Male	Unknown	Treatment failure	First line	Diagnosis	NR	Sputum	Negative	NTM
31.	41	Male	NR	NR	New			Sputum	Negative	NTM
32.	20	Female	Unknown	NR	First line	Follow up	3	Sputum	Negative	NTM
33.	38	Female	Unknown	Return after default	First line	Follow up	11	Sputum	Negative	NTM
34.	24	Female	Reactive	New case		Follow up	0	Sputum	Negative	NTM
35.	23	Female	Non-Reactive	New case	New	Follow up	15	Sputum	Negative	NTM
36.	42	Male	Unknown	Relapse case	First line	Follow up	13	Sputum	Negative	NTM
37.	43	Male		Relapse case	First line	Follow up	7	Sputum	Negative	NTM
38.		Male	Non-Reactive	New case	NR	Follow up	20	Sputum	Negative	NTM
39.	32	Female	Unknown	Relapse case	First line	Follow up	21	Sputum	Negative	NTM
40.	60	Male	Non-Reactive	New case	First line	Diagnosis	NR	Sputum	Scanty	NTM
41.	70	Male	Unknown	New case	First line	Follow up	4	Sputum	Negative	NTM
42.	22	Male	NR	NR	NR	Follow up	19	Sputum	Negative	NTM
43.		Male	Non-Reactive	Relapse case	second line	Follow up	2	Sputum	Negative	NTM
44.	15	Male	Non-Reactive	NR	First line	Follow up	5	Sputum	Negative	NTM
45.	30	Male	Non-Reactive	Relapse case	First line	Follow up	7	Sputum	Negative	NTM
46.	50	Female	NR	Relapse case	second line	Follow up	15	Sputum	Negative	NTM
47.	49	Male	Reactive	Relapse case	First line		0	Sputum	Positive +1	NTM
48.	52	Female	Non-Reactive		NR	NR	0	Sputum	Positive +1	NTM
49.	41	Male	Unknown	New case	First line	NR	NR	Sputum	Negative	NTM
50.	31	Male	NR	New case	NR	Diagnosis	NR	Sputum	Positive +2	NTM
51.	38	Male	Reactive	NR	First line	Follow up	10	Sputum	Negative	NTM
52.	13	Female	Non-Reactive	NR		Follow up	0	Sputum	Positive +2	NTM
53.	5	Male	Non-Reactive	Relapse case	second line	Follow up	5	Sputum	Negative	NTM
54.	5	Male	Non-Reactive	Relapse case	second line	Follow up	6	Sputum	Negative	NTM
55.	30	Male	NR	Relapse case	second line	Follow up	3	Sputum	Negative	NTM
56.	32	Male	NR	Treatment failure	NR	Follow up	17	Sputum	Negative	NTM

57.	14	Female	Unknown	New case	NR	Follow up	18	Sputum	Negative	NTM
58.	25	Male	NR	NR	First line	Follow up	NR	Sputum	Negative	NTM
59.	12	NR	NR	NR		Follow up	24	Sputum	Negative	NTM
60.	28	Male	Reactive	Relapse case	second line	Follow up	4	Sputum	Negative	NTM

NR: Not recorded.

Appendix 5. Quality control reads of sequenced isolates



Supplementary Figure 1. Sequence quality score plots of the 56 MTBC isolates, Addis Ababa, 2021. (A: the quality score per sequence B: mean sequence quality score, C: G-C content.)

Appendix 6. Data collection tools

ቅጥያ ሀ፡ ስለጥናቱ ጠቅላላ መረጃ እና የተሳታፊ ስምምነት መግለጫ ቅጽ

የጥንቱርዕስ፡ የሳንባ ቲቢ አምጭ ህዋስ (ማይኮባክቴሪያ) የዘር ልዩነትና መድሃኒት የመለማመድ ሁኔታ አዲስ አበባ ዉስጥ በሚገኙ ቲቢ በአክታ ቲቢ ያልተገኘባቸዉ የቲቢ ህሙማን መካክል

የጥናቱባለቤት፤ አለም አለማየሁ

የስራሁኔታ፤ በአ.አ.ዩ፣የተፈጥሮ ሳይንስ ኮሌጅ በማይክሮብያል ሴሉላር እና ሞሎኩላር ባዮሎጂ የት/ት ክፍል በአፕላይድ ማይክሮባዮሎጂ ስትሪም የሶስተኛ አመት የሶስተኛ ዲግሪ ተማሪ

ስልክቁጥር፡ +251979989713/ + 251916120451

ለዚህ ጥናት ድጋፍ ያደረጉ/ ስፖንሰር፤ አርማወር ሃንሰን የምርምር ኢንስቲቲዩት እና አዲስ አበባ ዩኒቨርሲቲ

አድራሻ፡ AHRI/ALERT AAERC secretariat : Tel: +251-11-34-81-28-9

AAU, CNS Ethical Review Board: Tel 251-5-95-92-16

እንደምንዋሉ/አደሩ?

በዚህ አህሪ እና አአዩ አስተባባሪነት በሚደረገው ጥናት ዉስጥ እንዲሳተፉ ፈቃደኝነትዎን እየጠየቅን ነው። እባክዎትን የትኛዉንም ዉሳኔ ከመወሰንዎ በፊት ቀጥሎ የቀረበዉን መረጃ ጊዜ ወስደው በደንብ ያንብቡ።ስለጥናቱ ማነኛዉም አይነት ጥያቄ ካለዎት ሳያመነቱ ይጠይቁ።

እኔ በአዲስ አበባ ዩኒቨሪስቲ በማይክሮቢያል ሴሉላር እና ሞሊኪዉር ባይሎጅ የትምህርት ክፍል ውስጥ የፒኤችዲ ተማሪ ለሆነችዉ አለም አለማየሁ እናየጥናት ቡድኑ "የሳንባ ቲቢ አምጭ ህዋስ (ማይኮባክቴሪያ) የዘር ልዩነትና መድሃኒት የመለማመድ ሁኔታ አዲስ አበባ ዉስጥ በሚገኙ ቲቢ በአክታ ቲቢ ያልተገኘባቸዉ የቲቢ ህሙማን መካክል" በሚል ርእስ ለሚያደርጉት ጥናት በመረጃ ሰብሳቢነት እየስራሁ ነው።በዚህ ጥናት ተሳታፊ እንዲሆኑ የእርስዎን የስምምነት ፍቃድ በአክብሮት እጠዪቃለሁ:: ቀጥሎ የቀረበውን ስለጥናቱና በጥናቱ ውስጥ ስለሚኖርዎት ተሳትፎ በተመለከተ መረጃ ልሰጦት እወዳለሁ::

መግቢያ፡በአክታ ቲቢ ያልተገኘበት የቲቢ በሽታ ማለት የሳንባ ቲቢ ሆኖ በማይክሮስኮፕ የክታምርመራ ዉጤት የቲቢ አምጭ ሕዋስ ያልተገኘበት ነገር ግን ህመምተኛው ባሳየው የህመሙ ምልክቶች እና የራጅ ምርመራ ዉጤት እንዲሁም በለሎች አጋዥ የላቦራቶሪ ምርመራዎች ተመስርቶ የሚታከም የሳንባ ቲቢ አይነት ነው።በኢትዮጵያ ከአጠቃላይ የሳንባ ቲቢ ስርጭት ይህ የቲቢ አይነት 50 % ይይዛል፡በአክታ ቲቢ ያልተገኘበት የሳንባ ቲቢ አይነት ህመምተኞች ትክክለኛዉን ህክምና /መድሃኒት በወቅቱ እንዳያገኙ ማድረግ በቲቢ በሽታ ቁጥጥር ላይ ትልቅ ተግዳሮት እየሆነ ነዉ።

ስለሆነም ይህ ጥናት የዚህን ቲቢ አምጭ ህዋሰ የዘር ልዩነት እና መደሃኒት የመለማመድ ሁኔታ ለማጥናት አላማ አድርጓል። በተጨማሪም ጥናቱ የታካሚዉን የሃኪም ምርመራ፣የማይክሮባዮሎጂና ኢሚዩኖሎጂ ዉጤቶችን ቲቢ በአክታ ባልተገኘባቸዉና

ቲቢበአክታ በተገኝባቸዉ የቲቢ ህሙማን መካከል ንጽጽራዊ መረጃ ያመነጭል። በዚህ ጥናት 518 አዋቂ የቲቢ ህሙማንን ለማሳተፍ ታቅዷል።ይህ ጥናት በአዲስ አበባ ዩኒቨርስቲ፣ የተፈጥሮ ሳይንስ ኮሌጅ የምርምር ስነምግባር ገምጋሚ ቦርድ፣በአርማወር ሃንሰ ንየምርምር ኢንስቲቲዩት የምርምር ስነምግባር ገምጋሚ ኮሚቴ እና በአዲስ አበባ ጤና ቢሮ የምርምር ስነ ምግባር ገምጋሚ ኮሚቴ፡ የምርምር ስነምግባር ማረጋገጫ አግኝቷል።ጥናቱ እስከሚጠናቀቅ ድረስ የአአዩ. እና አህሪ ድጋፍና ቁጥጥር አዪለየዉም።

የመረጃውአሰባሰብ ቅደም ተከተል/አካሄድ: የምርመራዎ ዉጤት/ሁኔታ ቲቢ በአክታ ቲቢ ያልተገኝባቸዉ የቲቢ ዐይነት / ቲቢ በአክታ የተገኝባቸዉ የቲቢ ዐይነት (ለማነጻጸሪያነት) ሰለሆነ በዚህ ጥናት እንዲሳተፉ እየጋበዝኖት ነዉ።በዚህ ጥናት ዉስጥ ለመሳተፍ ፈቃደኛ የሚሆኑ ከሆነ ይህንን የስምምነት ቅጽ መረዳት እና መፈረም ይኖርቦታል። በስምምነቱ መሰረት አንድ የሾርባ ማንኪያ (10-15) ሚ.ሊ የሚሆን ሁለት የአክታ ናሙና እና አንድ የሻይ ማንኪያ (5 ሚ. ሊ.) የደምና ሙና ይሰጣሉ።ሁሉም የላቦራቶሪ ምርመራዎች ከክፍያ ነጻ ናቸው።ከዚህ በተጨማሪ ከቲቢ በሽታ ተጋላጭነት ጋር ተያያዥ የሆኑ ጥያቄዎች ይጠየቃሉ። ቃለ መጠይቁ 10- 15 ደቂቃ የሚወስድ ሲሆን ጊዜ ሰጥተው እንዲተባበሩኝ እጠይቆታለሁ።መረጃውን የምሰበስበው ቀደም ብለው የተጻፉ ጥያቄዎችን ሳነብለወት ለጥያቄዎቹ በሚሰጡኝ ትክክለኛ መልስ ይሆናል።እንዲሁም ሌሎች የህክምና መረጃዎችዎ እና የራጅ ዉጤትዎ ለጥናቱ ግብአት ይሆናሉ። የኤች. አይ. ቪ ምርመራ እንዲደረግልዎ የሚስማሙ ከሆነ የቅድመ-ምርመራ እና የድህረ-ምርመራ የምክር አገልግሎት በባለሙያዎች አማካኝነት ይሰጦታል።

በጥናቱመሳተፍየሚያመጣውአደጋና የምቾትመጓደል: የዚህ ጥናት አካል ሆነው በመሳተፍዎ የሚደርስብዎት ችግር በጣም ትንሽ ነዉ።የደም ናሙና ከክንድዎ በሚወሰድበት ጊዜ የምቾት መጓደል ሊሰማዎት ችላል።።ከዚህ ሌላ የተወሰኑ ደቂቃዎችን ካለዎት ጊዜ ላይ ለቃለ መጠይቁ ስለሚወስድብዎት ሊሰላቹ ይችላሉ።

በጥናቱመሳተፍየሚያስገኝውጥቅም ፤በዚህ ጥናት በመሳተፍዎ የሚገኝ ቀጥተኛ ጥቅም አይኖርም።ነገር ግን በላቦራቶሪ ውጤት ላይ ተመስርቶ ተጨማሪ ህክምና እና እንክብካቤ ይመቻችሎታል።ይህ ጥናት በተጨማሪም የቲቢ አምጭ ህዋስን መድህኒት የመለማመድ ሁኔታ እና በማኅበረሰብ ዉስጥ የመዛመት እና የመቆየት ጠባይን ለማወቅ ያስችላል።ይህም ለወደፊቱ የቲቢ በሽታን ለመከላከልና ለመከላከል እንዲሁም በወቅቱ ለማከም የሚረዱ ስትራተጂዎችን ለማዉጣት ይጠቅማል።

ክፍያ/ ማበረታቻ ፤ በዚህ ጥናት በመሳተፍዎ ገንዘብ ክፊያ ወዪም የአይነት ማበረታቻ አይኖርም።

የጥናቱንሚስጥራዊነትስለመጠበቅ፤ ማነኛዉም የእርስዎን ማንነት የሚገልጽ መርጃዎች ሚስጢራዊነት የተጠበቀ ነው መረጃዎምቹ ምስጢራዊ ቁጥር በመስጠት ይያዛሉ እንጂ ስምዎ አይጻፍበትም በተጨማሪም የዚህ ጥናት ተሳታፊዎች መረጃ በአንድ ላይ ሆኖ የሚገለጽ እንጅ የአንድ ተሳታፊ መረጃ ብቻ ተነጥሎ አይቀርብም።ከጤና ባለሙያው እና ከተመራማሪዋ በስተቀር የትኛው ሚስጢራዊ ቁጥር የየትኛው ስም እንደሆነ ማን ምማወቅ አይችልም።

የጥናቱተሳታፊዎችመብት፤ በዚህ ጥናት የመሳተፍም ሆነ ያለመሳተፍ ሙሉ መብት አለዎት።ይህም በዚህም ሆነ በሌላ የጤና ተቋም ከሚያገኙት ማነኛዉም አገልግሎት ጋር የሚያገናኘው ነገር የለም፡፤እንዲሁም በማነኛዉም ሰዐት ጥናቱን አቋርጠው መዉጣት ይችላሉ።ይህም በጤና ተቋሙ የመገልገል መብትዎትን አያሳጣዎትም።

ማሳሰቢያ፤ ግልጽ ያልሆነ ወይም ማንኛውንም ጥያቄ መጠየቅ ከፈለጉ ለመመለስ ፈቃደኛ ስለሆን የሚከተለዉን አድራሻ መጠቀም ይችላሉ።

የጥናቱ ዋና ተመራማሪ አድራሻ፤ አለም አለማየሁ፡ በሞባይል ስልክ ቁጥር፤ 0916120451

ቅጥያ ለ፡ የሳንባ ቲቢ ላለባቸው ተሳታፊዎች የተዘጋጀ የፈቃደኝነ ት ማረጋገጫቅጽ

የጥንቱ ርዕስ፡ የሳንባ ቲቢ አምጭ ህዋስ (ማይኮባክቴሪያ) የዘር ልዩነትና መድሃኒት የመለማመድ ሁኔታ አዲስ አበባዉ ስጥ በሚገኙ ቲቢ በአክታ ቲቢ ያልተገኘባቸዉ የቲቢ ህሙማን መካከል

እኔ ስሜ ከዚህ በታች የተገለጸው የተሳታፊዎች መረጃ ቅጽ አንብቤአለሁ /ተነቦልኛል። ይህ ምርምር ''በቲቢ አማጭ ህዋስ የዘር ልዩነትና መድሃኒት የመለማመድ ሁኔታ አዲስ አበባ ዉስጥ በሚገኙ ቲቢ በአክታ ያልተገኘባቸው የቲቢ ህሙማን መካከል'' መሆኑን ተረድቻለሁ።ለጥናቱም ሁለት የአክታ ወይም \ እና የደም ናሙና እንደምሰጥ፤ እንዲሁም ቃለ መጠይቅ እንደማደርግ ተጠይቄአለሁ።በዚህ ጥናት በመሳተፌ የማገኘው ቀጥተኛ ጥቅም እንደማይኖር ነገርግን በላቦራቶሪ ውጤት ላይ ተመስርቶ ተጨማሪ ህክምና እና እንክብካቤ እንደሚመቻችልኝ ተረድቻለሁ የሁሉም መረጃዎች ሚስጥራዊነት ሙሉ በሙሉ እንደሚጠበቅ ተነግሮኛል። የመሳተፍ እንዲሁም ያለመሳተፍ ጉዳይ የራሴ ውሳኔ እንደሆነ በማንኛውም ጊዜ ጥናቱን የማቋረጥ መብት ያለኝ መሆኑን እና ይህም በእኔ ላይ የሚያደርሰው ምንም ችግር እንደሌለ ተነግሮኛል።ይህንን የስምምነት ማረጋገጫ ቅጽ ከመፈረሜ በፊት በቂ ጊዜ ወስጄ እንዳስብብት እድል ተሰጥቶኛል።በመሆኑም በዚህ ጥናት ለመሳተፍ በሙሉ ፈቃድ መወሰኔን በፊርማዬ አረጋግጣለሁ።

የተሳታፊ ፊርማ_____ ቀን: _____

የመረጃ ሰብሳቢ ስምና ፊርማ _____ ቀን_____

የእማኝ ፊርማ 1. _____ቀን _____

2. _____ ቀን_____

ማሳሰብያ፡ ይህ ሰምምነት የሚደረገዉ ናሙና በሚሰበስበዉ ሰዉ ባለበት መሆን አለበት

Annex 2. Data collection formats

Data collection formats for studying mycobacterial genetic diversity and drug resistance pattern among smear negative PTB patients

Part I.

A. Patient Enrolment assessment form

1. Patients Code ☐☐☐☐☐☐☐☐
2. Date of ☐☐☐☐☐☐ enrolment:(dd/mm/y)
3. CLINIC Card Number ☐☐☐☐☐
4. TB type SPPTB ☐ SNPTB ☐

B. Enrolment Acceptance Criteria

Inclusion criteria: Y N NA

- Willing to consent to study ☐☐☐
- Aged ≥18 years ☐☐☐
- Newly diagnosed TB patients ☐☐☐
- Patient sign Informed Consent: Yes ☐ No ☐
- Date Informed Consent signed: (dd/mm/yy) __ __/__ __/__ __ EC

Final dicition : Patient Included ☐ Patient Excluded ☐

Part II

Demographic data

1. Gender : M ☐ F ☐
2. Age ☐☐
3. Residence: Urban ☐ Rural ☐
4. Where do you live ? In my owen house ☐ With relatives ☐
   In elderly care center ☐ In prison ☐ On the Strait ☐
   In rehablitation center ☐ Other, specify ----------
5. Occupation : Daily labourer ☐ Farmer ☐ Student ☐ House wife ☐ ☐
   Merchant Governm☐t employee s☐ employed ☐
   Unemployed other----------
6. Educational level: Illitrate ☐ Read & writ local language ☐ ☐
   Primary ☐ Secondary ☐ Teritiery
7. Monthly income (ETB) ☐

Part III

A. Patient's symptoms

Cough Y ☐ N ☐ Duration at enrollm (tick one) mo ☐ wk ☐ day ☐

Score Severity *(intermediate scores per interviewer's discretion)* ☐☐

1 point: mild (infrequent, intermittent, shallow cough)

5 points: moderate (day cough, every two hours)

10 points: severe (day/night cough, frequent--every 15-30 minutes, deep, associated with pain or vomiting or gasping for breath between coughs)

Sputum Y ☐ N ☐ If yes, amount per day:Tea spoon (5cc) ☐ table ☐ spoon (15cc)

Sputum container (30cc) ☐ Ethiopian coffee cup (200cc) ☐

More than Ethiopian coffee cup (> 200cc) ☐

Blood Sputum Y ☐ N ☐ If yes, amount per day: ☐ specks only ☐ teaspoon (5cc) tablespoon (15cc) ☐ Sputum container (30cc) ☐

Ethiopian coffee cup(>200cc) ☐ More than Ethiopian coffee cup (>200cc) ☐

Night sweats (soak linen) Y N ☐ ☐

Chest pain Y N ☐ ☐

if YES, score severity:*(intermediate scores per interviewer's discretion)* ☐☐

10 points: worst pain in my life, can't function

7 points: Not worst pain but very difficult to function

5 points: Modereate pain, distracting, can function with some difficulty

3 points: Mild pain, able to functi

1 point: barely noticable pain

- Fever Y ☐ N ☐ If yes, How many days ☐☐
- Loss of weight Y ☐ N ☐ If yes, estimate weight loss (kg) ☐☐
- Shortness of breath Y ☐ N ☐
- If yes, Class I ☐ Class II ☐ Class III ☐ Class IV ☐

Amended 1994 Update of NYHA classification of functional capacity

Class I. Patients with no limitation of physical activity. Ordinary physical activity does not cause undue dyspnea.

Class II. Patients with slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity (climbing stairs, cleaning house) results in dyspnea.

Class III. Patients with marked limitation of physical activity. They are comfortable at rest. Minor activity (making bed, getting dressed) causes dyspnea .

Patient History

1. Medical history (Use back of page if inadequate space)

a.Ever had TB before? Y ☐ Unknown ☐

If YES, did you treated for TB Y ☐ N ☐ Unknown ☐

b. Underlying co-morbid condition	**Y**	**N**
Diabetes mellitus	☐	☐
Malignancy	☐	☐
COPD	☐	☐
Use of corticosteroid	☐	☐
End-stage renal disease	☐	☐
Liver cirrhosis	☐	☐
AIDS	☐	☐

Other, Specify----------

3. Patients HIV status Posetive ☐ Negative ☐

4. Histry of antibiotic exposure Y ☐ N ☐ Unknown ☐

If yes , for how many days did you take the antibiotics ----------days

If yes , indicate name of antibiotics ----------

Part IV: Substance use

A. History of cigarette smoking

1. Never smoked ☐
2. Currently smoking ☐
3. Smoked in the past but stopped ☐ Date stopped if known:

(EC dd/mm/yy, leave blank if unknown) ___/___/___

If precise date unknown, estimate how long ago: ____ years, _____ months ago

If currently smoking or smoked in the past, type of smoking

	Y	N	Average no of cigarette/pipes smoked/day
Cigarettes	☐	☐	_____
Home made cigarettes	☐	☐	_____
Pipe Tobacco	☐	☐	_____

If smoking history, how long have/had you smoked ? _____Years

B. Drug use: (eg chat, cannibis, etc)

Never used drugs ☐

I stopped using drugs ☐ Date stopped (EC dd/mm/yy, leave blank if unknown) __/ __/ __

If precise date unknown, estimate how long ago: ____ years, _____ months ago

I currently use drugs Y☐ N☐

	If yes, use:	Y	N	
Fill out info at right if drug user	Chat	☐	☐	---------- days/week
	Cannibis	☐	☐	---------- days/week
	Shisha	☐	☐	---------- days/week
	Other (specify) _____			---------- days/week

C. Alcohol use: Do you drink alcohol?

1. Never ☐
2. I use alcohol ☐
3. Previousy used > 6 months ago ☐

If you have used alcohol, indicate which beverage? If yes, check frequency

	Y	N	
Tej	☐	☐	----------days/week
Tella-	☐	☐	----------days/week
Beer	☐	☐	----------days/week
Whiskey/ Hard liquor	☐	☐	----------days/week
Wine	☐	☐	----------days/week
Arake	☐	☐	----------days/week

Part V

A. Examination (Clinician)

Temp (°C): ☐☐ PR: ☐☐ RR: ☐☐

(If subject temp is 37.0 write 37.0 not 37)

BCG scar Y ☐ N ☐

Anthropometry

Karnofsky score: ☐☐☐ **%**

Weight: ☐☐

Height: ☐☐☐ cm

BMI: Database Unit ☐☐.☐ (kg/m²)

Karnofsky score:

- 100% - normal, no complaints, no signs of disease
- 90% - capable of normal activity, few symptoms or signs of disease
- 80% - normal activity with some difficulty, some symptoms or signs
- 70% - caring for self, not capable of normal activity or work
- 60% - requiring some help, can take care of most personal requirements
- 50% - requires help often, requires frequent medical care
- 40% - disabled, requires special cd help

B. Pertinent signs of disease

	Y	N
Capchexia	☐	☐
Dyspnea	☐	☐
Lymphadenopathy	☐	☐
Abdominal tenderness	☐	☐
Ascites	☐	☐
Abnormal genitourinary findings (eg orchitis)	☐	☐
Abnormal skin findings	☐	☐
Bone (eg spine) tenderness	☐	☐
Abnormal heart sounds	☐	☐
Audible breath sounds (eg wheezing)	☐	☐
Clubbing	☐	☐
Pallor	☐	☐
Other	☐	☐

If other, describe:_____

C. Positive findings on auscultation:

a. crepitation (fine or course)	☐	☐
b. bronchial breath sounds	☐	☐
c. Decreased air entry	☐	☐
d. pleural friction rub	☐	☐
e. Other findings	☐	☐

If yes, specify: _____

Part VI

CXR Reading Report : Date performed: (dd/mm/yy) ___/___/___

Findings. tick all that apply

Yes	No	
☐	☐	**Infiltrate or consolidation**
☐	☐	**Any Cavity lesion**
☐	☐	**Nodule with poorly-defined margins**
☐	☐	**Pleural effusion**
☐	☐	**Hilar or Mediastinal Lymphadenopathy**
☐	☐	**Discrete fibrotic scar or linear opacity g**
☐	☐	**Discrete nodule(s) without calcification**
☐	☐	**Discrete fibrotic scar with volume loss or retr**

If Other, specify: _____

TB diagnosis confirmed by (tick all that apply)

X-ray ☐ AFB Smear ☐ Xpert ☐ Culture ☐ Expert dicition ☐ Other----------

ESR result ----------mm/hr

Sputum AFS Staining result: AFS + Y ☐ N ☐ If yes, **1+** ☐ **2+** ☐ **3+** ☐ Not Indicated ☐

AFS – Y ☐ N ☐

Gene Xpert result:	MTB not Detected	Y ☐	N ☐
	Detected MTB only	Y ☐	N ☐
	Detected MTB+ RRD	Y ☐	N ☐
	Indeterminate result	Y ☐	N ☐
	Not available	Y ☐	N ☐

Samples Collected:	**Yes**	**No**	**Date**
Sputum 1	☐	☐	__ __/__ __/__ _
Sputum 2	☐	☐	__ __/__ __/__ _
BAL	☐	☐	__ __/__ __/__ _
Blood /Serum (2 tube)	☐	☐	__ __/__ __/__ _

Signature PI/CoI

Appendix 7. List of publications and papers presented

Publications from the dissertation

1. *Alemayehu, A., Wassie, L., Alemayehu, D. H., Adnew, B., Neway, S., Tefera, D. A., Ayalew, S., Hailu, E., Ayele, S., Seyoum, B., Bobosha, K., Abebe, M., Aseffa, A., Petros, B., Howe, R. (2024). Genotypes and drug resistance pattern of Mycobacterium tuberculosis complex among clinically diagnosed pulmonary tuberculosis patients. Front Public Health, 12; https://doi.org/10.3389/fpubh.2024.1420685*

2. *Alemayehu, A., Wassie, L., Neway, S., Ayele, S., Bobosha, K., Aseffa, A., Petros B and & Howe, R. (2024). Clinical and imaging characteristics of smear negative pulmonary tuberculosis patients: a comparative study. Pulmonary Medicen, 2024. doi: 10.1155/2024/2182088*

3. *Alemayehu A, Kebede A, Neway S, Tesfaye E, Zerihun B, Getu M and Petros B (2022). A glimpse into the genotype and clinical importance of non-tuberculous mycobacteria among pulmonary tuberculosis patients: The case of Ethiopia. PLoSONE 17(9): e027; https://doi.org/10.1371/journal.pone.0275159*

Papers Presented

1. TB research advisory council (TRAC) 2024, Addis Ababa Ethiopia: Whole genome based genetic diversity of *M. tuberculosis* complex isolated from smear negative tuberculosis patients: a comparative study
2. TB research advisory council (TRAC) 2023, Gondar Ethiopia: Clinical and imaging characteristics of smear negative pulmonary tuberculosis patients: a comparative study (Won best abstract Award)
3. The Union World Conference on Lung Health 2022, France (Virtual): Clinical and imaging characteristics of smear negative pulmonary tuberculosis patients: a comparative study.
4. The Union World Conference on Lung Health 2023, France (Virtual): Culture negative pulmonary tuberculosis patient's clinical and imaging findings: a comparative study form TB endemic setting.
5. Keystone symposia 2020 (Virtual): Frequency and isolation of nontuberculous mycobacteria from tuberculosis patients in Ethiopia.