Addis Ababa University

School of Graduate Studies

College of Natural and Computational Sciences

Department of Microbial, Cellular and Molecular Biology

Molecular Epidemiology and Drug Resistance Analysis of *Mycobacterium tuberculosis* isolates from Central, Eastern and Southeastern Ethiopia

By

Mulualem Agonafir

A DISSERTATION SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

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DECLARATION

I the undersigned declare that this PhD Thesis is my own original work and has not been presented for a degree in any other university and all sources of materials used for the Thesis have been duly acknowledged.

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ABSTRACT

Molecular Epidemiology and Drug Resistance Analysis of *Mycobacterium tuberculosis isolates* from the Central, Eastern and Southeastern Ethiopia

By

Mulualem Agonafir

Supervisors: Prof. Gurja Belay, Prof. Bernard Fourie and Dr. Adey Feleke

Understanding the prevalent lineages, drug resistance mutations, and transmission dynamics of *Mycobacterium tuberculosis* complex (MTBC) across diverse regions is crucial for designing effective tuberculosis (TB) control strategies. In Ethiopia, comprehensive studies on the molecular epidemiology and drug resistance of TB are scarce, with existing research focused on specific regions like Addis Ababa and the north/southwestern parts. This study aimed to assess genetic diversity, transmission patterns, and drug resistance mutations among *Mycobacterium tuberculosis complex* (MTBC) isolates in central, eastern, and southeastern Ethiopia. Conducted between August 2018 and January 2019, it involved 232 culture-positive MTBC isolates from pulmonary TB patients referred to Adama and Harar TB reference laboratories. Spoligotyping identified prevalent lineages and sub-lineages, revealing a diverse population structure with six major lineages. The Euro-American (Lineage 4) and East-African-Indian (Lineage 3) lineages were dominant, comprising nearly 95% of isolates. High genetic diversity was observed, with 77 distinct spoligotype patterns. Dominant spoligotypes included SIT149/T3_ETH, SIT53/T1, SIT21/CAS1_Kili, and SIT41/Turkey, with a rare Beijing spoligotype (SIT541) detected in eastern Ethiopia. Strain clustering was significantly associated with individuals aged 25-34 years. Genotypic drug susceptibility testing identified mutations conferring resistance to rifampicin (RIF) and isoniazid (INH) in nearly 40% of isolates. Mutations for resistance to fluoroquinolones (FLQs) and second-line injectable drugs (SLIDs) were less frequent, observed in around 9% and 4% of isolates, respectively. Dominant mutations included S531L in *rpoB* for RIF, S315T in *katG* for INH, A90V in *gyrA* for FLQs, and WT1 in *rrs* for SLIDs. WGS of multidrug-resistant (MDR) MTBC isolates supported these findings, revealing detailed information on specific drug resistance mutations for an expanded list of drugs. The Euro-American (Lineage 4) and East-African-Indian (Lineage 3) remained the most prevalent genotypes among MDR MTBC isolates in East Ethiopia. Core genome Multi-locus Sequence Typing (cgMLST) analysis revealed recent MDR-TB transmission events in 46.7% of clustered isolates, with a high proportion from Diredawa city, suggesting localized transmission. One cluster was the Beijing sub-lineage of East Asian (Lineage 2), known for high transmission and drug resistance. In conclusion, this study highlights the diverse genetic structure and significant drug resistance mutations of MTBC isolates in central, eastern, and southeastern Ethiopia. The findings emphasize the dominance of specific lineages and sub-lineages, the presence of diverse mutations, and localized transmission hotspots. This underscores the need for tailored control strategies and comprehensive molecular surveillance to effectively address TB and MDR-TB in Ethiopia.

Keywords: Drug resistance mutations, Ethiopia, Genetic diversity, Line probe assays, Multidrug-resistant tuberculosis, *Mycobacterium tuberculosis* complex, Spoligotyping, Whole genome sequencing

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LIST OF ABBREVIATIONS

cgMLST	Core genome Multi-locus Sequence Typing
CRISPR	Clustered Regularly Interspersed Short Palindromic Repeats
DR	Drug-resistant
EA	Euro-American
EAI	East African Indian
EAS	East-Asian
FLQs	Fluoroquinolones
FMOH	Federal Ministry of Health
HIV	Human Immunodeficiency Virus
INH	Isoniazid
MDR-TB	Multidrug-resistant TB
MIRU	Mycobacterial interspersed repetitive units
MOHE	Ministry of Health-Ethiopia
MST	Minimum Spanning Tree
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MUT	Mutant
NGS	Next-Generation Sequencing
pre-XDR-TB	Pre-extensively drug-resistant TB
PZA	Pyrazinamide
QRDR	Quinolone-resistance-determining Region
RFLP	Restriction Fragment Length Polymorphism

RIF	Rifampicin
RRDR	Rifampicin Resistance-determining Region
RR-TB	Rifampicin-resistant TB
SIT	Shared International Type
SLIDs	Second-line Injectable Drugs
SNP	Single Nucleotide Polymorphism
TB	Tuberculosis
VNTR	Variable Number of Tandem Repeats
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild Type
XDR-TB	Extensively drug-resistant TB

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1. Background

TB, which is caused by MTBC, is a treatable, preventable and curable disease (Reid et al., 2019), yet it remains one of the most important cause of death and poor health globally (WHO, 2022a). The END TB strategy envisions a world free of TB by 2035, with a target of less than one incident case per million population (Matteelli et al., 2018). This strategy represents a paradigm shift from TB control to elimination, emphasizing the importance of addressing the reservoir of latent TB infections to prevent future cases (Matteelli & Alffenaar, 2022). According to recent estimates (Houben & Dodd, 2016), roughly 1.7 billion individuals, which equates to nearly 23% of the world's population, are believed to be latently infected with *Mycobacterium tuberculosis* (MTB). This substantial pool of infected individuals serves as a source for future TB cases, with projections indicating that by 2030 and 2050, 16.3 and 8.3 active TB cases per 100,000 population will emerge from this pool, respectively (Houben & Dodd, 2016).

In 2021, it was estimated that approximately 10.6 million people were infected with TB, with 87% of those infections occurring in the 30 countries with the highest TB burden, primarily in the WHO regions of South-East Asia, Africa and the Western-Pacific. Of those cases, 56.5% were men, 6.7% were people living with HIV, and 11% were children (WHO, 2022a).

Although curative antituberculosis therapy has been available for nearly half a century, the inappropriate and inadequate treatment of TB, coupled with unchecked transmission of MTB, has led to alarming levels of drug-resistant (DR) TB (Daley & Caminero, 2018).

Subsequently, efforts to control TB have been significantly impacted by the emergence of various DR-TB strains, including MDR-TB, pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB). MDR-TB is resistant to INH and RIF, pre-XDR-TB has additional resistance to a fluoroquinolone, and XDR-TB has resistance to RIF, plus any fluoroquinolone, plus at least one further priority A drug (bedaquiline or linezolid) (Viney et al., 2021; WHO, 2022e). According to WHO, there were an estimated 450,000 naïve cases of MDR-TB and an additional 78,000 cases of rifampicin-resistant TB (RR-TB) worldwide in 2021. The proportion of new TB cases and previously treated individuals with MDR/RR-TB was estimated to be 3.6% and 18%, respectively. India (26%), the Russian Federation (8.5%), and Pakistan (7.9%) accounted for the majority of these cases (WHO, 2022a).

In Ethiopia, TB is one of the leading public health issues, with over a quarter of affected patients going undetected (MOHE, 2021). Ethiopia is among the 30 high-burden TB and HIV associated TB high-burden countries with 143000 people infected with TB and 21,000 deaths in 2021 (WHO, 2022a), indicating that TB remains a leading cause of mortality in the country. Drug resistant TB remains a significant challenge in Ethiopia's response to TB. According to the results of the third national drug resistance survey, which was completed in 2019, the MDR-TB prevalence among new patients was 1.03%, while among previously treated patients it was 6.52% (MOHE, 2021).

Studies indicate that most cases of DR-TB occur due to transmission rather than initial acquisition during TB treatment (Naidoo et al., 2024; Pečerska et al., 2021; Shah et al., 2017; Sharma et al., 2017; Yang et al., 2017). Therefore, early detection of anti-TB drug resistance and prompt initiation of effective treatment are crucial to reducing the spread of

drug-resistant TB (Zhang & Yew, 2009). Recent advances in molecular diagnostics have enabled the introduction of diagnostic tools including the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) and line probe assays (Hain Lifescience, Nehren, Germany), which are used for the rapid detection of both MTB and common mutations that confer drug resistance to key anti-TB drugs (Dlamini et al., 2019; Mahomed et al., 2017; WHO, 2022b). Unlike the commonly used commercial molecular tools, whole genome sequencing (WGS) has proven superior in identifying mutations beyond the classic resistance determining regions, which are not covered by commercial genotypic assays (Lam et al., 2021) and holds the potential to answer many unresolved questions (Satta et al., 2018).

Genotyping of MTBC has become an essential public health tool, enabling researchers and TB control programs to grasp how specific strains emerge and spread, as well as gauge the influence of genetic diversity on the outcomes of TB infection and disease (Comas & Gagneux, 2009). Common genotyping methods that are often utilized include IS6110 restriction fragment length polymorphism (RFLP) typing (Otal et al., 1991), spoligotyping (Kamerbeek et al., 1997) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) analysis (Supply et al., 2006). As next-generation sequencing (NGS) technologies continue to advance and become more affordable, WGS typing is increasingly performed to fully utilize the available genetic information. Effective outbreak investigations, transmission dynamics, and global epidemiological surveillance of TB disease rely heavily on these typing methods (Merker et al., 2017). When compared with the classical typing methods, WGS stands out for its robustness and high resolution (Coscolla & Gagneux, 2014).

Human TB is primarily caused by members of the MTBC, which have over 99% genomic similarity (Gagneux, 2018) and are organized into distinct population structures based on their geographical distribution (Ssengooba & Chin, 2020). Until recently, the human-adapted species of MTBC, *M. tuberculosis sensu stricto* and *M. africanum* (MA), were categorized into seven phylogenetic lineages (L1-L7): L1 (Indo-Oceanic/IO); L2 (East-Asian/EAS); L3 (East African Indian/EAI); L4 (Euro-American/EA); L5 and L6 (MA West Africa 1 and West Africa 2, respectively) and L7 (Ethiopia/ETH) (Brites et al., 2018; Coll et al., 2014; Couvin et al., 2020). Two additional lineages were identified in recent years: L8, or *M. tuberculosis* from the African Great Lakes (Ngabonziza et al., 2020); and L9, a new strain of *M. africanum* (Coscolla et al., 2021). These lineages exhibit variations in propensity to acquire drug resistance, biological fitness and virulence (Comas et al., 2013; Stucki et al., 2016; Warner et al., 2015). In general, L2 and L4 are the most ubiquitous, while L5, L6 and L7 are limited to specific African regions (Coscolla & Gagneux, 2014). The new lineages, L8 and L9 were also identified from African regions (Coscolla et al., 2021; Ngabonziza et al., 2020), but are yet to be investigated further. Research conducted in various regions of Ethiopia has revealed the existence of numerous lineage varieties, with L4 and L3 being the most prevalent (Abebe et al., 2019; Damena et al., 2019; Tadesse et al., 2017).

The current study characterized MTBC isolates from pulmonary TB patients in central, eastern and southeastern Ethiopia to identify the predominant lineages, transmission dynamics and drug resistance charactersitcs.

1.2. Rationale of the study

TB remains a major global health challenge, with Ethiopia experiencing a high burden of the disease. Understanding the genetic diversity of MTBC strains is critical for effective TB control strategies (Osei-Wusu et al., 2022). Various lineages of MTBC have been identified worldwide, each with unique characteristics and distribution patterns. Despite the growing body of research on MTBC diversity in Ethiopia, much of the existing literature focuses on specific regions, such as Addis Ababa and north/southwestern areas (Mekonnen et al., 2019; Tulu & Ameni, 2018). Consequently, there is limited information about the genetic diversity of MTBC strains in central, eastern, and southeastern Ethiopia. Moreover, the emergence of MDR-TB poses a significant threat to TB control efforts globally. In Ethiopia, although progress has been made in TB control, MDR-TB continues to be a substantial challenge (MOHE, 2021). There are few studies on molecular characterization of MDR-TB. Rapid diagnostic technologies have advanced the detection of drug resistance, yet comprehensive data on the mutations conferring resistance to key anti-TB drugs across different regions of Ethiopia is lacking.

Therefore, conducting the current study holds substantial significance for several reasons, impacting both local and global TB control efforts. By examining the genetic diversity of MTBC strains in central, eastern, and southeastern Ethiopia, this study will provide a more comprehensive understanding of the epidemiology of TB in these regions. This knowledge is crucial for identifying and tracking the spread of specific MTB lineages, which can inform targeted interventions and control measures.

The study's insights into the population structure and genetic diversity of MTB will be instrumental in refining clinical management practices, allowing clinicians to make more informed decisions about treatment regimens based on the local prevalence of specific MTB strains and their associated drug resistance patterns. By identifying the frequency and types of mutations conferring resistance to key anti-TB drugs, the study will contribute to improving diagnostic accuracy and treatment efficacy.

Understanding the genetic makeup and distribution of MTB strains as well as detailed characterization of MDR MTBC isolates, can help tailor TB control strategies to the specific needs of different regions, aiding in optimizing TB surveillance, diagnosis, and treatment protocols for more effective and efficient control efforts.

In general, the comprehensive data generated from this study will provide a robust evidence base for policymakers to develop and implement TB control policies tailored to the specific epidemiological landscape of Ethiopia, ensuring that resources are allocated effectively and that interventions are designed to have the maximum impact.

1.3. Research questions

1. What is the genetic diversity of MTBC isolates in Central, Eastern, and Southeastern Ethiopia?
2. Which MTBC lineages and sub-lineages are predominant in these regions, and are there any geographic variations?
3. What is the level of clustering among MTBC isolates, and are there specific factors associated with clustering?
4. What is the proportion and distribution of mutations associated with RIF, INH, FLQs and SLIDs resistance among MTBC isolates from pulmonary TB patients in central, southeastern, and eastern Ethiopia?
5. What is the significance of the presence of unknown mutations?
6. What are the main strains of MTBC driving the transmission dynamics of MDR-TB?
7. What are the specific mutations related to MDR MTBC isolates?

1.4. Objectives

1.4.1. General Objective

The general objective of this study was to assess the genetic diversity, transmission patterns, and drug resistance mutations among MTBC isolates in central, eastern, and southeastern Ethiopia using genotypic tools.

1.4.2. Specific Objectives

- To describe the genetic diversity of MTBC isolates in central, eastern and southeastern Ethiopia.
- To assess mutations conferring drug resistance to RIF, INH, FLQs and SLIDs in MTB isolates from central, eastern and southeastern Ethiopia
- To characterize MDR MTBC isolates in central, eastern and southeastern Ethiopia using spoligotyping and WGS to understand their genetic diversity, transmission patterns, and drug resistance mutations against a wider range of anti-TB drugs.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1. The *Mycobacterium tuberculosis* complex

The MTBC, which comprises several closely related species, can be classified into those that have evolved to thrive within human hosts and those capable of disseminating and transmitting across a broad range of animal species, both wild and domesticated (Malone & Gordon, 2017). These include *M. tuberculosis and M. africanum* which are human-adapted; and the animal-adapted species *M. bovis* (bovids), *M. microti* (voles), *M. orygis* (oryxes, gazelles, rhesus monkeys of South Asian origin), *M. pinnipedii* (seals and sea lions), *M. caprae* (goats and sheep), *M. mungi* (banded mongooses), dassie bacillus (hyrax) and *M. suricattae* (meercats) (Alexander et al., 2010; Aranaz, 2003; Bañuls et al., 2015; Coscolla & Gagneux, 2014; Cousins et al., 2003; Mostowy et al., 2004; Parsons et al., 2013; Simner et al., 2016; Van Ingen et al., 2012).

The *M. canettii* strains, also referred as smooth tubercle bacilli, have more genomic variability and distinct colony morphology compared to the MTBC strains. They were formerly classified as MTBC but now are seen as a separate clade due to their distinct phenotypic and genomic features, which differ from classical MTBC members (Supply & Brosch, 2017).

The members of MTBC have almost identical (>99%) nucleotide sequences and completely identical 16S rRNA sequences (Bañuls et al., 2015). Although MTBC members exhibit identical characteristics, there are substantial disparities in their biochemistry, disease patterns in animals, host ranges, morphology, drug susceptibility data, geographic distribution, and epidemiological patterns (Forbes et al., 2018).

MTB is the most clinically relevant among the MTBC species, as it is the primary cause of TB in humans (Rodríguez-Hernández et al., 2020). The bacterium belongs to the class actinomycetes, order actinomycetales, family mycobacteriaceae and genus Mycobacterium (Shinnick & Good, 1994). It is an aerobic, non-spore forming, nonmotile and slow-growing pathogen with generation time of 18-24 h (Moon et al., 2015). The optimal temperature for its growth is 35-37^{0}C, and it thrives when grown on substrates that utilize glycerol as a carbon source and ammonia or amino acids as nitrogen sources (Fitzgerald et al., 2015; Simner et al., 2016).

The cell wall of MTB, which is characterized by a prominent presence of lipids and carbohydrates (Figure 1), plays a vital role in the bacterium's survival and pathogenicity by providing a barrier against drugs. This barrier is formed by a thick layer of peptidoglycan and an outer membrane mainly composed of various lipopolysaccharides, fatty acids, glycolipids and wax esters, which are unique features of cell wall of mycobacteria (Abrahams & Besra, 2018; Jankute et al., 2015; Vilchèze, 2020). The inner membrane, which is constructed from phospholipids and contains glycolipids that protrude into the periplasm, is further surrounded by the core cell wall. This core wall consists of three primary components: arabinogalactan, peptidoglycan and mycolic acids. Additionally, the mycolate layer is intercalated with solvent-extractable lipids including inert waxes and glycophospholipids, which form the outer membrane. Finally, the outermost layer of the bacterium is the capsule that primarily contains proteins and polysaccharides (Abrahams & Besra, 2018).

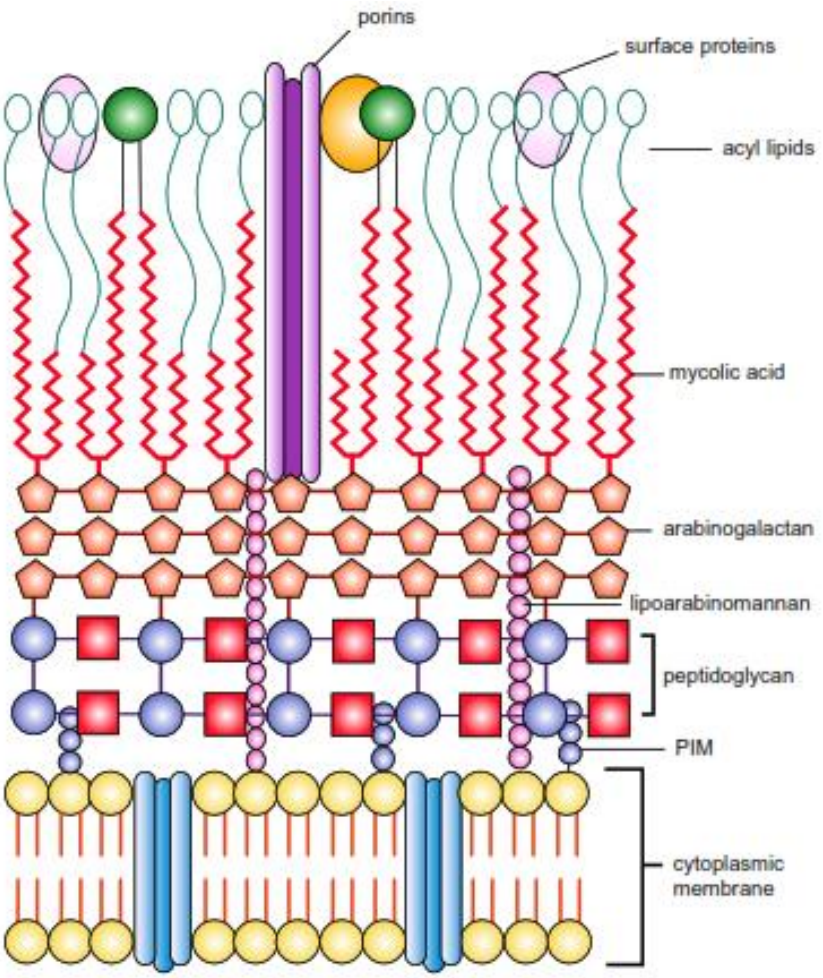

Figure 1: Schematic diagram of *Mycobacterium* cell wall.

The arrangement includes cytoplasmic membrane, periplasm, cell wall compounds (peptidoglycan, arabinogalactan, mycolic acids) and associated lipids, and outer layer (called capsule). Similar to the outer membrane of the gram-negative cell wall, porins are required to transport small hydrophilic molecules through the outer membrane of the acid-fast cell wall. PIM, Phosphatidyl-myo-inositol mannosides (Velayati & Farnia, 2017).

2.2. Forms of TB Disease

TB in humans manifests primarily as pulmonary TB, which is the most common form, but it can also disseminate to other organs, leading to extrapulmonary TB (EPTB). Pulmonary TB affects the lungs, while EPTB can affect various parts of the body, including lymphatic system, central nervous system (as in miliary and meningeal TB), genitourinary system, bones and joints, and other tissues (Ellner & Jacobson, 2019; Ramana, 2014; Yang & Kong, 2015). Miliary TB is a severe form characterized by widespread dissemination of the bacteria throughout the body, often leading to high mortality rates, especially when diagnosis and treatment are delayed (Ellner & Jacobson, 2019; Ray et al., 2013). TB meningitis, another form of EPTB, is particularly serious, with significant neurological consequences and high mortality (Ellner & Jacobson, 2019). Additionally, genitourinary TB (GUTB) is noted as a frequent form of EPTB (Ramana, 2014).

2.3. Transmission and Pathogenesis of *Mycobacterium tuberculosis*

MTB is an airborne pathogen that is transmitted from person to person through the inhalation of contaminated droplet nuclei (1-5 μm in diameter) which are small enough to remain airborne and spread through air currents (Donald et al., 2018; Marimani et al., 2018; Patterson & Wood, 2019). The transmission of MTB from an index case to a contact in a shared air space depends on the sputum bacillary concentration and cough frequency, duration of exposure, intensity of exposure and the strain's virulence (Dheda et al., 2010; Jones-López et al., 2013).

MTB pathogenesis involves a complex interplay between the bacteria and the human immune system, details in (Ahmad, 2011; Alsayed & Gunosewoyo, 2023; CDC, 2019;

Kanabalan et al., 2021). As shown in figure 2, infection begins when the inhaled droplet nuclei containing MTB bacilli travel via mouth or nose, the upper respiratory tract, bronchi and reach the alveoli in the lung where the bacteria are engulfed by alveolar macrophages and other phagocytotic cells (Alsayed & Gunosewoyo, 2023; Kanabalan et al., 2021). Some bacilli get eliminated, but others escape and multiply intracellularly which then cause systemic dissemination reaching different parts of the body (Ahmad, 2011). The body's immune system usually gets activated in 2-8 weeks, halting the multiplication of the bacteria and preventing further dissemination (CDC, 2019). Immune cells such as macrophages, T cells, neutrophils and inflammatory lymphocytes form granulomas, which encase the necrotic tissue and limit the spread of tubercle bacilli. The majority of the bacilli within these granulomas are killed, and the progression of the disease is halted (Ahmad, 2011). However, some individuals may not completely eliminate the bacilli as MTB can evade the microbicidal mechanisms of phagocytic cells. Subsequently, around 5-10% of individuals will develop active TB, while in 90-95% of individuals infection remains latent (Rook et al., 2005). When the immune response is disrupted, the dormant bacilli reactivate and cause active TB in approximately 5-10% of immunocompetent people with latent TB, later in their life (Rook et al., 2005; Sia & Rengarajan, 2019). HIV co-infection is the main contributing factor for TB reactivation in latently infected people with an increased annual risk of 10% (CDC, 2019). Other risk factors such as malnutrition, young age, diabetes, indoor air pollution, alcohol consumption, use of immunosuppressive drugs, solid organ transplantation and tobacco smoke, significantly contribute to the progression from infection to active TB disease (Ferrara et al., 2012; Goletti & Martineau, 2021; Narasimhan et al., 2013). Characterized by weight loss, toxicity of tumor-necrosis factor, cavitation,

and fibrosis, progressive disease is marked by the eventual opening of cavities into the bronchi, which facilitates the spread of TB through aerosols during coughing (Rook et al., 2005).

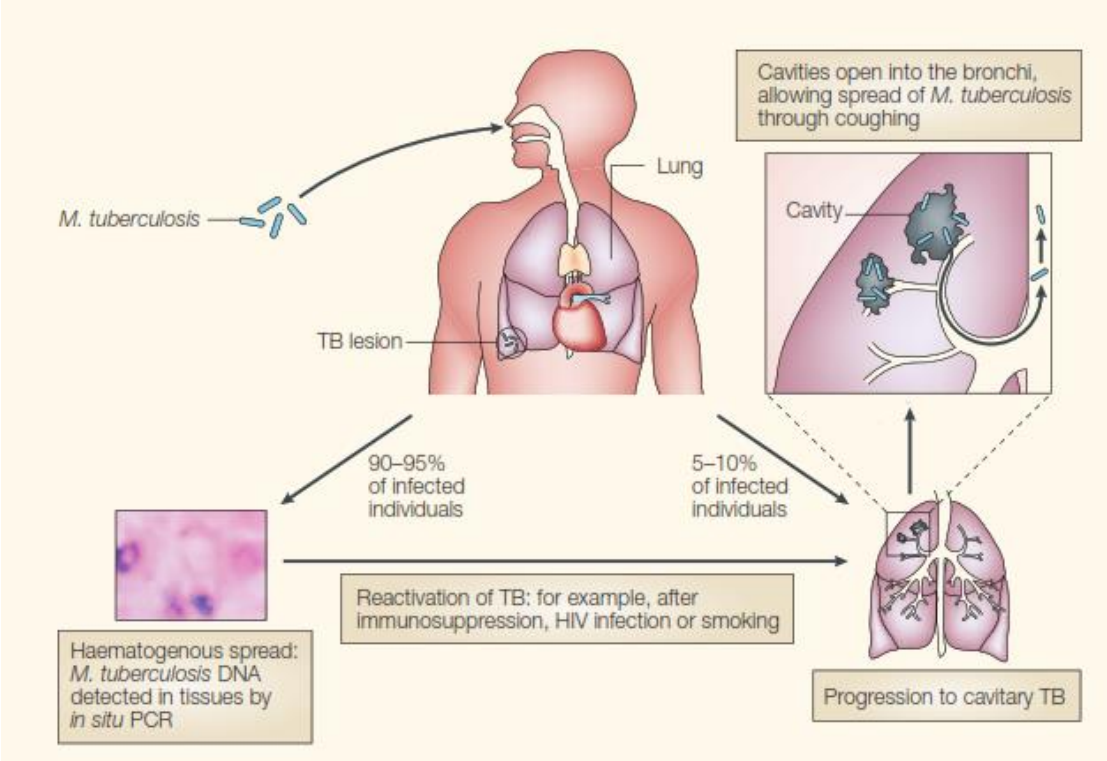

Figure 2: Phases of infection with *Mycobacterium tuberculosis* (Rook et al., 2005)

2.4. The impact of COVID-19 on TB care and prevention efforts

The COVID-19 pandemic has disrupted TB control programs, leading to a decline in TB notification rates and posing challenges to the implementation of TB elimination strategies (Krishnamoorthy & Ramachandran, 2022; Razak et al., 2023). Patients with TB have faced barriers to diagnosis and treatment due to changes in health services, and the pandemic exacerbated the difficulties in accessing healthcare (Syamsir et al., 2023). The economic repercussions of COVID-19 have also impacted the WHO's End TB Strategy goal of

eliminating catastrophic costs for TB-affected households by 2030 (Fuady et al., 2021). Interestingly, despite these setbacks, some innovative strategies have emerged. Digitalization of service delivery, telemedicine, and public engagement initiatives have shown promise in reviving TB notification rates (Krishnamoorthy & Ramachandran, 2022). Moreover, the integration of COVID-19 and TB screening, the use of telehealth, and international support have been suggested as ways to mitigate the pandemic's impact on TB management (Caren et al., 2022).

2.5. Vaccine development and prospects

Given the limitations of the current BCG vaccine in preventing the ongoing global TB epidemic, the development of new TB vaccines is critical. Promising advances have been made with various vaccine candidates, including recombinant BCG strains, DNA-based vaccines, and subunit vaccines with novel adjuvants, showing efficacy in preclinical models and progressing to human clinical studies (Brennan et al., 2004; Da Costa et al., 2023). Notably, recent phase 2b clinical trials have shown efficacy signals for preventing TB infection and disease, which has reinvigorated the field and provided hope for more effective vaccines (Sable et al., 2019). However, there are challenges to overcome, such as complex regulatory issues, the need for a better understanding of TB immunology, and the identification of correlates of protection to streamline vaccine development (Brennan et al., 2004; Z. Hu et al., 2022). Additionally, while some candidates have shown promise, such as M72/AS01E and Vaccae, others have had disappointing results, highlighting the need for continued research and embracing risk in vaccine development (Chetty & Chetty, 2022; Scriba et al., 2020).

2.6. Genotyping tools for MTBC

One of the significant breakthroughs in the field of TB research has been the advent of molecular techniques that allow the identification and monitoring of individual MTB strains. These methods have enabled the establishment of molecular epidemiology as a new field of study, which has furthered our knowledge on the transmission patterns of MTB and supplemented classical epidemiology (Burgos et al., 2004). The primary objective of TB epidemiology is to determine the origin of infection and delineate the routes through which it disseminates in the environment. To attain this goal, it is crucial to possess the capacity to discern and monitor particular strains of MTB (Jagielski, van Ingen, et al., 2014).

In the past, prior to the development of molecular typing or genotyping, MTB isolates were classified at the species level through phenotypic and biochemical examination (Amlerova et al., 2018). Furthermore, anecdotal correlations and observational data were used to understand TB transmission (Mathema et al., 2006). However, the application of molecular typing techniques for MTBC in recent decades has considerably augmented our understanding of mycobacterial biology and management of TB (Jagielski, van Ingen, et al., 2014). These methods have proven effective in differentiating between closely and distantly related strains, detecting new infections and reactivated cases, as well as identifying the predominant genotypes present (Asare et al., 2021).

To investigate the transmission dynamics of TB, polymorphisms present in the mycobacterial genome are exploited and are used as genetic markers. In principle, a DNA marker must exhibit enough polymorphism to differentiate between non-related isolates

and it must be sufficiently stable to establish a connection between related isolates, which are indeed genetically linked. The method employed to assess the genetic polymorphism must be straightforward, cost-effective, and capable of generating timely results. Furthermore, the presentation of results in a format that can be easily shared between laboratories is a crucial requirement (Kato-Maeda et al., 2011).

The most commonly used genotyping techniques for investigating the genetic diversity and transmission patterns of MTBC include IS6110 restriction fragment length polymorphism (RFLP), spoligotyping, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) and WGS.

2.6.1. IS6110 restriction fragment length polymorphism (RFLP)

The RFLP method, which relies on the variability in number and position of insertion element IS6110: MTBC-specific mobile genetic element with a highly conserved sequence (Kato-Maeda et al., 2011), was among the pioneering typing techniques employed for MTB (Barbier & Wirth, 2017). This method was the first to be standardized molecular typing (Van Soolingen et al., 1994) and is widely recognized as the reference standard for molecular epidemiologic studies of TB (Ei et al., 2016; Schürch & Van Soolingen, 2012; Warren et al., 2009).

As depicted in Figure 3, this technique entails the utilization of the restriction enzyme *PvuII* to cleave the DNA, followed by agarose electrophoresis and Southern blot hybridization to separate the resulting fragments. The presence of IS6110 is determined by identifying fragments containing the element, and the resulting band pattern, which can be compared

among different isolates using a computer software, signifies a distinct genotype (Amlerova et al., 2018).

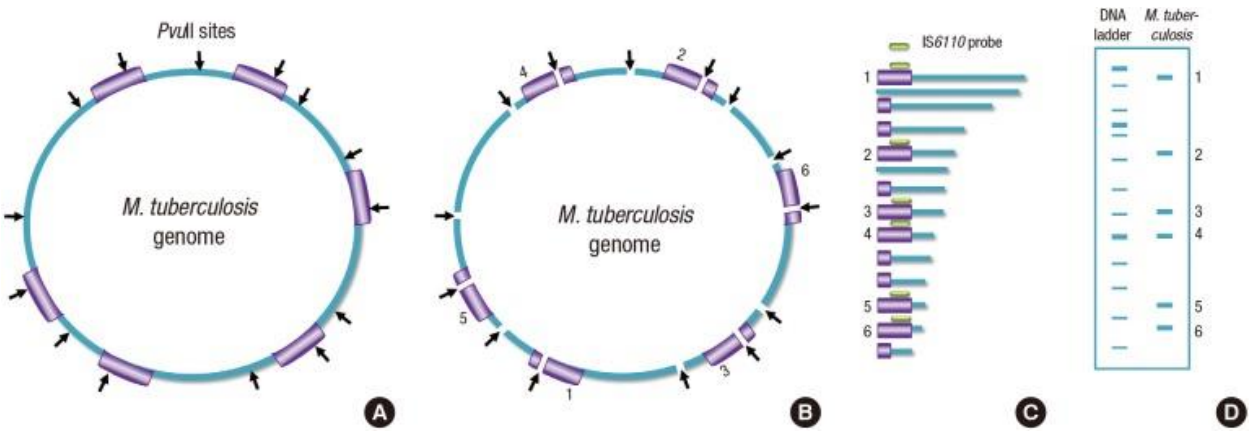

Figure 3: Method of RFLP.

(A) The genome of *M. tuberculosis* with the insertion segment IS6110 and the *PvuII* cleavage sites. (B) Digestion of the whole genome with *PvuII*. (C) DNA segments of different sizes determined by gel electrophoresis are transferred onto a nitrocellulose membrane followed by hybridization. (D) Visualized fragments, which represent a single copy of IS6110 surrounded by flanking DNA of different lengths (Ei et al., 2016).

Although IS6110 RFLP typing has proven to be a valuable epidemiological tool, it is also hindered by four major limitations: first, the technique is relatively complex and requires a certain level of technical expertise; second, a significant amount of highly purified DNA from the slow-growing organism is necessary; third, the resulting data are presented as banding patterns, which can make comparison between laboratories unreliable or at least challenging; and fourth, the technique's ability to discriminate between strains is poor when the IS6110 element is present at low copy number (less than 5) (Warren et al., 2009). Consequently, the need for a large quantity of DNA (5-10µg) which requires cultivation for several weeks, and the exceedingly laborious procedures render this method unsuitable

for epidemiological inquiries during outbreaks, where a swift outcome is critical, and for extensive population-based studies (Moström et al., 2002).

2.6.2. Spacer oligonucleotide typing or Spoligotyping

Spoligotyping is a PCR-based genotyping technique that is extensively utilized for MTBC isolates. This methodology is predicated on the polymorphism of the direct repeat (DR) locus, which is also called clustered regularly interspersed short palindromic repeats (CRISPR) locus, found in MTBC isolates (Couvin et al., 2020; Jansen et al., 2002; Kamerbeek et al., 1997). The DR locus contains multiple direct variable repeats (DVRs), each comprising a 36 bp-DR and a non-repetitive 34 to 41 bp long spacer sequence (van Embden et al., 2000) (figure 4). The presence or absence of particular spacers are used to discriminate MTB strains (Groenen et al., 1993). At present, 94 unique spacer sequences have been identified, out of which 43 are used in the first-generation spoligotyping for MTBC strains (van der Zanden et al., 2002; Van Embden et al., 1993). Of these, 37 are unique to MTB (spacers 1-19, 22-32, and 37-43), while the remaining 6 (spacers 20-21 and 33-36) are utilized for analyzing *M. bovis* strains (van der Zanden et al., 2002).

Figure 4: Direct repeat locus (fragment).
43 spacers are used in spoligotyping assay (Kremer et al., 2002).

The entire direct repeat locus is subjected to PCR amplification, followed by hybridization to a membrane that contains 43 oligonucleotides corresponding to the spacers found in

MTB H37Rv and MTB BCG. The presence or absence of each of the 43 spacers in the direct repeat region of the analyzed isolate will be indicated by a pattern of positive or negative hybridization signals. The hybridization signals are detected using chemiluminescence and visualized through autoradiography or the Luminex® technology (Cowan et al., 2004; Groenen et al., 1993; Kamerbeek et al., 1997; Kremer et al., 2002) (Figure 5).

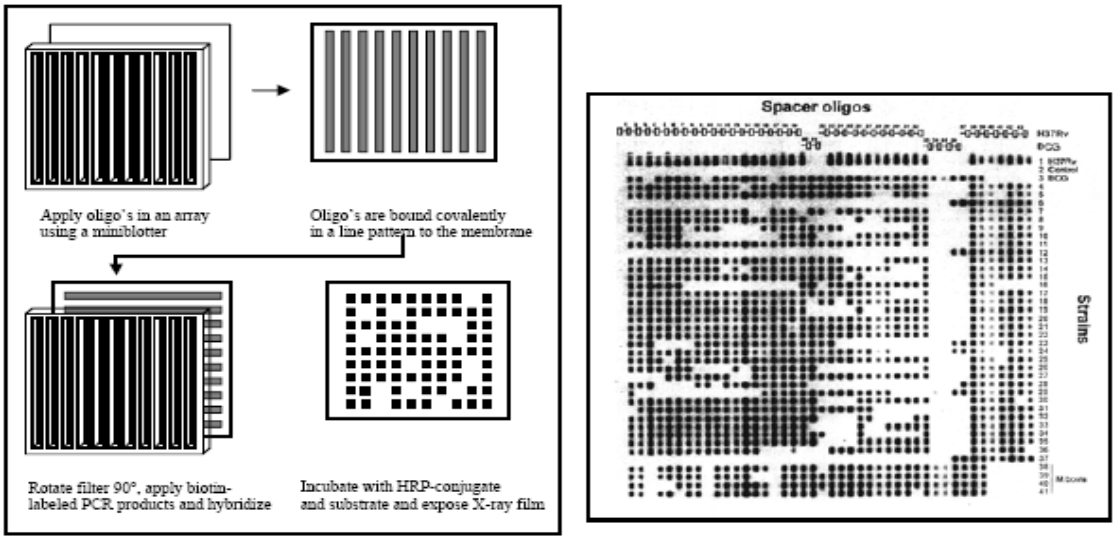

Figure 5: Overview of the spoligotyping method (Kremer et al., 2002).

The presence or absence of spacer is represented in a binary format, making it easy to interpret, computerize, and compare between different laboratories (Yin C. et al., 2023). This is reflected in international databases such as SpolDB4/SITVIT (Brudey et al., 2006; Demay et al., 2012), which describe 1,939 shared types and 3,370 orphan types from 39,295 MTBC isolates across 122 countries. SITVIT2, the updated version of SPOLDB4/SITVIT, was recently published and consists of 9,658 spoligotypes (corresponding to 103,856 clinical isolates) grouped into 3,851 shared types containing

98,049 clinical isolates and 5,807 orphan patterns collected from 131 countries (Couvin et al., 2019a).

Spoligotyping is a cost-effective, dependable, and prompt method (Brudey et al., 2006; Cowan et al., 2004; Yang & Gao, 2018) that differs from RFLP in its ability to directly analyze clinical samples (Goyal et al., 1997), nonviable cultures (Driscoll et al., 1999), Ziehl-Neelsen smear slides (Molina-Moya et al., 2018; van der Zanden et al., 1998) and paraffin-embedded tissue section (van der Zanden et al., 1998). It is also advantageous when used for isolates with few IS6110 copies (5 or less), as these isolates lack sufficient polymorphism for distinct identification via RFLP (van der Zanden et al., 2002). However, when utilized independently, spoligotyping is inadequate for epidemiological studies (De Viedma & Pérez-Lago, 2018). Moreover, this method may miss contaminated isolates or multi-strain infections in clinical samples (Jagielski et al., 2014).

2.6.3. Mycobacterial Interspersed Repeat Unit-Variable Number Tandem Repeats (MIRU-VNTR)

The MTB chromosome contains repeated sequences in tandem, which are polymorphic among different strains due to a variable number of tandem repeats (VNTR). These structures resemble minisatellites observed in eukaryotic cells (Coll & García de Viedma, 2018; Frothingham & Meeker-O'Connell, 1998; Mazars et al., 2001). Mycobacterial interspersed repetitive units (MIRU) are one example of VNTR which are tandem repeats of 46–101 bp dispersed across 41 loci throughout the MTB chromosome (Supply et al., 1997, 2000)). Several of these loci display variations in both copy number and repeat

length, which are essential components of the MIRU-VNTR typing technique in distinguishing isolates into unique genetic profiles (Kato-Maeda et al., 2011).

MIRU-VNTR is a technique that entails examining tandemly repeated sequences at various loci, which are amplified by primers that flank regions of each locus. Subsequently, the resulting PCR products are visualized by gel electrophoresis to determine the number of MIRU-VNTR copies that have been specifically targeted (Shamputa et al., 2010; Supply et al., 2006; Woodman et al., 2019) (Figure 6).

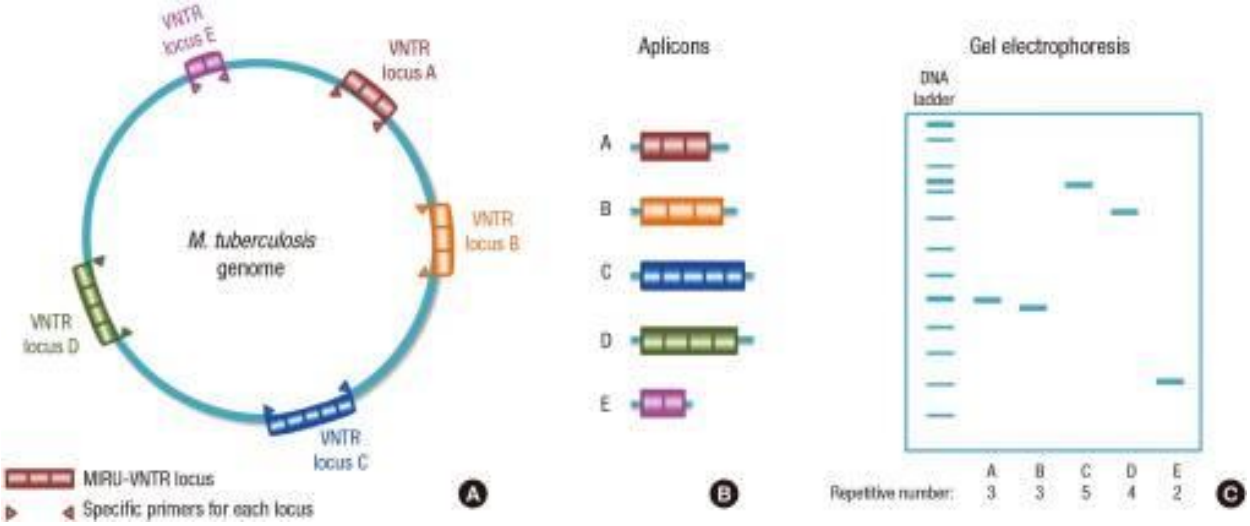

Figure 6: Principle of MIRU-VNTR genotyping.
(A) Multiple MIRU-VNTR loci amplified by specific primers for each locus. (B) Different sizes of amplicons after PCR. (C) Amplicons after gel electrophoresis with varying sizes that reflect the repetitive number of each VNTR locus (Ei et al., 2016).

The 24-locus MIRU-VNTR typing system is considered to be as effective as IS6110-RFLP profiling in terms of discriminatory power. As a result, it has been proposed as the new gold standard for molecular typing of MTBC strains (Jagielski et al., 2016; Schürch & Van Soolingen, 2012) and is currently widely used (Yang & Gao, 2018).

Advantages of MIRU-VNTR include high reproducibility, discriminatory power, ease of use and cost effectiveness (Anderson et al., 2014; Coll & De Viedma, 2018; Cowan et al., 2002; Kremer et al., 2005). The results of the method as digital MIRU-VNTR code facilitates easy data exportation, and global databases, such as https://www.miru-vntrplus.org/MIRU/index.faces (Weniger et al., 2010) and http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE (Demay et al., 2012) enable comparison of strains worldwide. Furthermore, unlike spoligotyping or RFLP, the ability of MIRU-VNTR in identifying multiple strains or clonal variants has led to a better understanding of mixed or polyclonal infections in a population (Micheni et al., 2022; Navarro et al., 2011; Pérez-Lago et al., 2011).

2.6.4. Whole genome sequencing (WGS)

WGS has significantly advanced our understanding of the MTBC, revealing a wealth of single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) that contribute to the genetic diversity of the pathogen (Stucki, 2015; Stucki & Gagneux, 2013). The high accuracy of WGS in evaluating diversity among MTB isolates has shown that conventional genotyping methods, even those with better discriminatory power, such as the 24-loci MIRU-VNTR, are insufficient to accurately define clusters (De Viedma & Pérez-Lago, 2018). WGS offers several advantages over classical genotyping methods for MTBC typing. It provides higher discriminatory power, allowing for the construction of robust phylogenies and characterization of the population structure of the MTBC (Stucki, 2015). WGS also enables the investigation of strain micro-evolution, identification of superspreaders and transmission direction during outbreaks, an aspect that classical methods cannot resolve effectively (Roetzer et al., 2013; Walker et al., 2013). Additionally,

WGS is better equipped to accurately predict drug-resistant phenotypes throughout the genetic makeup of a microorganism (Katale et al., 2020).

The full potential of WGS can only be realized with improved accessibility and standardization; along with the development of user-friendly bioinformatics tools and comprehensive genomic databases (Dohál et al., 2020; Rivière et al., 2020; Takiff & Feo, 2015). For instance, the application of WGS gene-by-gene MLST methods in combination with Ridom SeqSphere software has yielded a more streamlined and user-friendly approach for resolving and comprehending outbreaks, compared to traditional WGS SNP mapping (Jones et al., 2019; Kohl et al., 2018). As WGS becomes more accessible and sequencing costs decrease, it is poised to become the gold standard for MTBC typing in molecular epidemiological studies (Amlerova et al., 2018; Schürch & Van Soolingen, 2012).

2.7. Lineages of human-adapted MTBC

Human-adapted members of the MTBC have undergone co-evolution with human populations, influenced by factors like host immune response and human demography (Brites & Gagneux, 2012). This co-evolution has led to genetic diversity and adaptation, resulting in mutations conferring drug resistance and phenotypic variations affecting virulence (Mvubu & Jacoby, 2023). Although MTBC species share over 99% genomic similarity (Gagneux, 2018), they exhibit variations in drug resistance acquisition, biological fitness and virulence (Comas et al., 2013; Stucki et al., 2016; Warner et al., 2015) as well as distinct population structures based on geographical distribution (Coscolla & Gagneux, 2014).

Initially categorized into seven phylogenetic lineages (L1-L7) (Brites et al., 2018; Coll et al., 2014; Couvin et al., 2020), two new lineages, L8 (Coscolla et al., 2021) and L9 (Ngabonziza et al., 2020) were identified recently (Figure 1). These 9 lineages are further classified into two clades (modern and ancient) based on the absence or presence of TbD1 deletion (Brosch et al., 2002; Mvubu & Jacoby, 2023). The modern clade, consisting of a monophyletic group encompassing EAS/L2, EAI/L3, and EA/L4, is distinguished by the absence of the TbD1 region (Brites & Gagneux, 2017; Koleske et al., 2023) which correlates with increased resistance to stress and hypoxia (Bottai et al., 2020). This clade is associated with global TB epidemics and enhanced virulence, as evidenced by increased replication rates in macrophages and induction of pro-inflammatory cytokines (Correa-Macedo et al., 2019; Romagnoli et al., 2018). The ancient clade with intact TbD1 genomic region, consists of IO/L1 and L5 through L9 (Mvubu & Jacoby, 2023). Compared to modern lineages, ancient lineages, while diverse, do not induce the same level of immune response, potentially affecting disease progression and transmission (Portevin et al., 2011). Additionally, while modern lineages are considered more virulent, ancient lineage IO/L1, have shown less susceptibility to anti-TB drug pretomanid (Bateson et al., 2022). This suggests that ancient lineages may possess unique genetic or metabolic traits that confer different responses to therapeutic agents (Bateson et al., 2022).

The ancient ETH/L7, recently identified in Woldia (Firdessa et al., 2013), is prevalent in northern Ethiopia (Comas et al., 2015; Nebenzahl-Guimaraes et al., 2016). It exhibits genetic heterogeneity and has been positioned phylogenetically between IO/L1 and the modern lineages EAI/L3, and EA/L4 (Nebenzahl-Guimaraes et al., 2016). ETH/L7 strains have also been associated with a longer patient delay in seeking treatment and exhibit

slower growth (Yimer et al., 2015). Investigations into the lipid metabolism of MTBC have revealed a diminished capacity of EA/L7 in synthesizing virulence-associated cell wall lipids relative to EAS/L2 and EA/L4 lineages, which may affect their virulence and transmissibility (Moopanar & Mvubu, 2020). Recent study also reported that ETH/L7 exhibit a stop-gain mutation in the mmaA3 gene, affecting cell wall lipid synthesis, potentially influencing host-pathogen interactions and its limited geographical distribution (Hailu et al., 2023). The widely studied L2 Beijing lineage is often highlighted for its association with more severe disease outcomes, enhanced transmissibility and drug resistance including outbreaks and the spread of MDR-TB (Domenech et al., 2017; Glynn et al., 2002; Merker et al., 2015; Mertaniasih et al., 2020). The findings discussed above underscore the complexity of MTBC and the importance of considering lineage-specific traits in the development of therapeutic strategies, diagnostics and vaccines (Brosch et al., 2020; Domenech et al., 2017; Furnham et al., 2021; Portevin et al., 2011).

Based on the breadth of the ecological niches, EA/L4 is further grouped as globally distributed generalists and geographically restricted specialists suggesting that different sub-lineages have adopted distinct strategies for survival and spread, influenced by human migration patterns (Coll et al., 2014; Stucki et al., 2016).

The geographical distribution of MTBC lineages is influenced by a combination of historical migration, local adaptation, and host-pathogen interactions (Brosch et al., 2002; Coscolla et al., 2013; Mvubu & Jacoby, 2023). In general, IO/L1 is prevalent in South Asia, EAS/L2 (including the Beijing strain) is widespread globally, EAI/L3 is successful in Northern and Eastern Africa and Southern Asia, EA/L4 has a global presence, Lineages 5 and 6 (MA West Africa 1 and 2) are primarily found in West Africa, and ETH/L7 is

associated with Ethiopia and Ethiopian immigrants (Blouin et al., 2012; Brudey et al., 2006; Coscolla & Gagneux, 2014; Demay et al., 2012; Firdessa et al., 2013; O'Neill et al., 2019; Shuaib et al., 2022). The recently identified L8 (Coscolla et al., 2021) and L9 (Ngabonziza et al., 2020) in Africa add to the complexity and diversity of the MTBC (Figure 7). Epidemiological studies in Ethiopia indicate the dominance of EA/L4 and EAI/L3 with significance presence of IO/L1 and ETH/L7 among Ethiopian patients (Mekonnen et al., 2019; Tulu & Ameni, 2018).

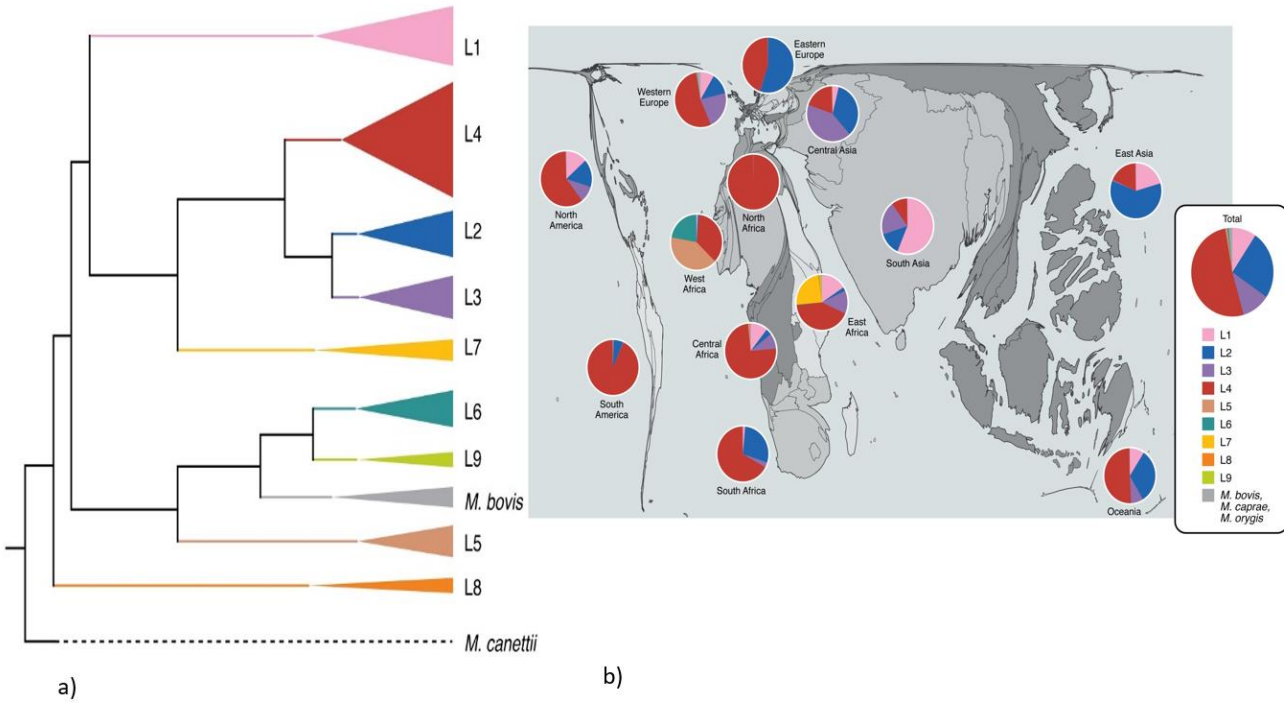

Figure 7: Phylogeny and distribution of the 9 lineages of MTB.

a) Simplified maximum likelihood phylogeny of the 9 lineages of MTB, as well as *M. bovis* strain that shares ancestry with L6 and the *M. canettii* outgroup strain used as a root. b) Global TB Burden Cartogram: Distribution of MTBC lineages (L1–L9) and animal-adapted MTBC: *M. bovis*, *M. caprae*, *M. orygis* (Koleske et al., 2023).

2.8. Molecular detection of drug resistance in TB

The conventional culture-based method for diagnosing TB and detecting drug resistance is a well-established approach that involves growing MTB from clinical specimens and testing its susceptibility to anti-TB drugs. This method, while accurate, is known for being time-consuming, often taking several weeks to produce results (Palomino et al., 2008). The delay in diagnosis and commencement of appropriate treatment poses risks to both the patient and the public due to the potential spread of drug-resistant TB (Kwaghe et al., 2014).

Molecular detection of drug resistance in TB is a critical component in the management and control of the disease, particularly in the face of MDR and XDR strains of MTB (Domínguez et al., 2023; Palomino & Martin, 2014). Increased understanding of the molecular mechanisms underlying resistance to anti-TB drugs over the past two decades has facilitated the creation of commercial genotypic assays for the prompt detection of drug resistance (Li et al., 2020; WHO, 2022b). These tools provide notable benefits for improving the management and monitoring of DR-TB programs. They facilitate early diagnosis, ensure standardized testing, and are adaptable to high-throughput environments. Moreover, these methods require fewer laboratory biosafety precautions, making them especially suitable for resource-constrained settings (Cirillo et al., 2017; WHO, 2014).

Currently, there are various commercial molecular assays used for detecting drug resistance in TB. Most of them are included in the list of WHO-recommended rapid diagnostics (WRDs) (WHO, 2021). Of these, the Xpert MTB/RIF cartridge-based system developed by Cepheid and the Hain line probe assays (LPA) produced by Hain Lifescience

are the two most commonly utilized molecular tests (Koch et al., 2018). As WRDs are tailored for specific drugs and focus on particular regions of the MTB genome, they have a constrained ability to identify a limited range of mutations (Cabibbe et al., 2018; Dlamini et al., 2019; Faksri et al., 2016; Witney et al., 2016). Conversely, WGS offers comprehensive insights into the genomic features that confer drug resistance, enabling the detection of specific mutations across the entire genome of the strain under investigation (Cohen et al., 2019; Witney et al., 2016).

The Xpert MTB/RIF and its more sensitive successor, the Xpert MTB/RIF Ultra, are primarily designed for detecting MTBC and RIF resistance (Pereira et al., 2020; Sharma et al., 2022). Studies indicate that the Xpert MTB/RIF Ultra assay exhibits higher sensitivity than the Xpert MTB/RIF assay, especially in paucibacillary sputum specimens and children suspected of having TB (Kolia-Diafouka et al., 2019; Yadav et al., 2021). In contrast, the Xpert MTB/XDR assay expands drug resistance detection capabilities to include isoniazid (INH) and second-line drugs such as fluoroquinolones (FLQ), ethionamide (ETO), and second-line injectable drugs (SLID). This expansion facilitates the rapid diagnosis and appropriate treatment of drug-resistant TB (Cao et al., 2021; Mvelase & Mlisana, 2022; Zhurilo et al., 2022).

GenoType MTBDR*plus* and GenoType MTBDR*sl* (Hain Lifescience GmbH, Nehren, Germany), hereafter referred to as MTBDR*plus* and MTBDR*sl*, are LPAs that provide rapid results within a five-hour timeframe, making them invaluable tools in clinical settings (Cirillo et al., 2017). These assays detect specific mutations linked to resistance against certain first and second-line drugs in smear positive samples and mycobacterial culture isolates (HAIN Lifescience, 2015a, 2015b). The MTBDR*plus* (v1 and v2) detect mutations

in the *rpoB* gene for RIF resistance and *katG/inhA* genes for INH resistance. MTBDR*plus* v1 was endorsed in 2008 by the WHO for rapid detection of MDR-TB in smear and culture positive isolates (WHO, 2016). The later version (MTBDR*plus* v2) can be used on smear negative specimens (Lin et al., 2022). Overall, MTBDR*plus* has consistently demonstrated high sensitivity and specificity in various studies for detecting RIF and INH resistance in MTBC. The sensitivity for RIF resistance ranges from 80% to 100%, and for INH, it varies from 82.7% to 100%. Specificity for both RIF and INH resistance detection is consistently reported at or near 100% in multiple studies (Abanda et al., 2017; Abdel Dayem et al., 2019; Bai et al., 2016; Benaissa et al., 2022; Lee et al., 2016). The variability in INH resistance sensitivity may be influenced by the genetic diversity of MTBC strains and the prevalence of specific resistance-conferring mutations in different populations (Omer et al., 2016; Maharjan et al., 2017).

The MTBDR*sl* has also two versions: version 1 and version 2. MTBDR*sl* v1 was the first commercial test designed to detect resistance to second-line anti-TB drugs. Subsequently, a few years after the release of MTBDR*sl* v1, MTBDR*sl* v2 was introduced, which included probes to detect mutations in genes that are linked to resistance to either fluoroquinolones or SLIDs (*gyrA* and *rrs* for version 1 and those genes plus *gyrB* and the *eis* promoter for version 2) (WHO, 2021). The MTBDR*sl* v2 assay demonstrates high specificity and variable sensitivity for different second-line anti-TB drugs across different regions and populations. For instance, sensitivity for fluoroquinolones ranged from 57.1% to 100%, and for second-line injectable drugs, it ranged from 47% to 100% across studies (Bouzouita et al., 2021; Jeong et al., 2016; Jin et al., 2013; Singh et al., 2013; Singh et al., 2021; Tekin et al., 2017; Zivanovic et al., 2012).

WGS, a cutting-edge molecular drug-susceptibility testing approach, examines the genome sequence of either cultured mycobacterial isolates or direct clinical samples (Hong et al., 2022; Murphy et al., 2023). WGS data offers insights into genetic variants or mutations linked with resistance to all presently employed antitubercular drugs. This information assists clinicians in making informed decisions regarding the selection of an appropriate treatment regimen for patients (Hirani et al., 2020a). WGS has high accuracy for first-line drugs like INH, RIF, EMB and PZA and has the potential to improve the detection of resistance to other drugs through the identification of novel mutations and lineage-associated resistance patterns (The CRyPTIC Consortium and the 100,000 Genomes Project, 2018; Wang et al., 2022; Xiao et al., 2023).

However, there are limitations associated with its implementation and accuracy. Firstly, WGS has a higher reagent cost compared to conventional phenotypic DST, which may limit its use in resource-constrained settings (Van Beek et al., 2019). Additionally, the sensitivity and specificity of WGS can vary depending on the drug being tested and the software tools used for data analysis, with some drugs like ethambutol and streptomycin showing lower concordance rates with phenotypic DST (Xiao et al., 2023). The turnaround time for WGS, although faster than culture-based assays, still requires several days, which may delay treatment decisions (Van Beek et al., 2019). Furthermore, WGS may not detect all resistance-conferring mutations, particularly those present as minority variants or in non-targeted regions of the genome (Charette et al., 2024). The presence of uncharacterized mutations can also lead to indeterminate results, necessitating further phenotypic testing (Walker et al., 2015). The performance of WGS also depends on the depth of sequencing

and the quality of the bioinformatics pipeline, which can lead to discrepancies in detecting certain mutations (Charette et al., 2024; Wang et al., 2022).

2.9. Treatment of active drug-susceptible and drug-resistant TB

The overarching goal of TB treatment is multifaceted, encompassing several key aims. Primarily, it is aimed at curing the individual patient by eliminating the MTB bacteria, thereby halting the transmission of the disease to others (Bhaskar et al., 2019). Additionally, there is a crucial objective to minimize the risk of developing drug resistance, which can arise from non-compliance with the prescribed treatment regimen (Bhaskar et al., 2019; Herawati et al., 2021). Furthermore, effective TB treatment endeavors to address and manage any adverse drug reactions that may occur, ensuring that the patient's quality of life is maintained or even enhanced during and after therapy (Chung-Delgado et al., 2011). To ensure proper administration and completion of TB treatment, WHO introduced Directly Observed Treatment, Short-Course (DOTS) (Behzadifar et al., 2015; WHO, 1997). Several countries, especially those in developing regions where the disease's burden is most pronounced, adopted this approach. While effective, the implementation of DOTS faces several challenges that need to be addressed to optimize its success (Behzadifar et al., 2015; Prudhivi et al., 2018).

The standard treatment regimen for active cases of drug-susceptible TB (DS-TB) typically involves a two-phase approach: intensive and continuation. The intensive phase consists of a four-drug regimen of INH, RIF, PZA, and EMB once daily for 2 months; followed by a continuation phase of INH and RIF daily for an additional 4 months (Horne & Nahid, 2021; WHO, 2022c). This regimen, established based on clinical trials conducted by the British

Medical Research Council (Fox et al., 1999), has been a mainstay in TB control programs for an extensive period. Recently, WHO recommended all-oral 4-month regimen containing INH, rifapentine (RPT), moxifloxacin (MFX) and PZA as an option for treating patients with DS-TB (WHO, 2022c).

The treatment of DR-TB presents significant challenges due to the resistance of MTB strains to first-line and certain second-line anti-TB drugs (Jain & Dixit, 2008; Seaworth & Griffith, 2017). For years, patients infected with DR-TB including RR/MDR-TB were treated using a long regimen (18-24 months) of second-line anti-TB drugs, recognized for their toxicity, reduced efficacy and higher costs (WHO, 2019). However, the treatment of DR-TB has significantly advanced in the last decade with the approval of new drugs like bedaquiline and pretomanid. This progress includes the most recent shift to short-course, injection-free regimens and the approval of 6-month treatment containing bedaquiline, pretomanid, linezolid and moxifloxacin (BPaLM) for RR/MDR-TB (Dookie et al., 2022). Ushering in a new era of patient-centered care, the conventional 18–24 months DR-TB treatment regimen has undergone a significant transformation. This redesigned approach provides flexibility, with treatment durations now ranging from 6 to 18 months, based on individual factors such as past TB episodes, drug intolerance, prior drug exposure and other factors (WHO, 2022d). While shorter regimens are expected to be the standard for most patients, roughly 9% of MDR-TB cases globally are estimated to still require longer treatments by 2026 (Gupta et al., 2024). The longer treatment regimen could involve a combination of drugs from Groups A, B, and C of the WHO classification system for DR-TB treatment. As shown in table 1, these groups classify drugs based on their effectiveness and potential side effects (WHO, 2020).

Table 1: WHO classification of anti-TB medicines for longer DR-TB regimens (WHO, 2020)

Groups	Medication	Abbreviation
Group A: Include all three medicines	Levofloxacin or	LFX
	moxifloxacin	MFX
	Bedaquiline	BDQ
	Linezolid	LZD
	Clofazimine	CFZ
Group B: Add one or both medicines	Cycloserine or	CS
	terizidone	TRD
Group C: Add to complete the regimen and when medicines from Groups A and B cannot be used	Ethambutol	EMB
	Delamanid/Pretomanid	DLM/PA
	Pyrazinamide	PZA
	Imipenem–cilastatin or	IMP-CLN
	meropenem	MPM
	Amikacin or streptomycin	AM or STM
	Ethionamide or	ETO
	prothionamide	PRO
	P-aminosalicylic acid	PAS

2.10. Molecular mechanisms behind drug-resistant TB

DR-TB poses a critical threat to global health, estimated to be responsible for 13% of all deaths attributable to antimicrobial resistance worldwide (Farhat et al., 2024). Hence, understanding the molecular mechanisms by which MTB develops resistance to anti-TB drugs holds immense value. This knowledge unlocks two critical avenues for combating TB: rapid diagnosis and targeted treatment. Understanding resistance mechanisms allows

for the development of rapid molecular diagnostic tests that enable healthcare professionals initiate appropriate treatment regimens promptly (Islam et al., 2017; Zhang & Yew, 2015a). Furthermore, this knowledge serves as a cornerstone for designing new drugs and treatment strategies. By targeting novel pathways or overcoming existing resistance mechanisms, these advancements can significantly improve patient outcomes (Da Silva & Palomino, 2011; Singh et al., 2020; Zhang & Yew, 2015b).

MTB is not known to exhibit horizontal gene transfer or mobile resistance elements, which are crucial mechanisms for the rapid spread of resistance (Nimmo et al., 2022). Instead, it primarily acquires resistance through mutations in genes encoding drug targets or activating enzymes. These mutations, often manifested as SNPs or INDELs (Allué-Guardia et al., 2021; Dookie et al., 2018; Tunstall et al., 2021), can come at a fitness cost for the bacteria, potentially reducing their virulence (Zhang & Yew, 2015b). Furthermore, while drug resistance in MTB often leads to a fitness cost, compensatory mutations can arise, restoring fitness and facilitating the transmission of resistant strains (Emane et al., 2021).

2.10.1. INH

INH, a pro-drug requiring activation by the *katG*-encoded catalase-peroxidase (Vilchèze & Jacobs, 2014), holds historical significance as the first drug demonstrating potent bactericidal activity against rapidly growing MTB *in vitro* and *in vivo* (Vilchèze & Jacobs, 2019). Mutations in several genes, including *fabG1*, *inhA*, *katG* and the *oxyR'-ahpC* intergenic region contribute to INH resistance. Among these, mutations within the *katG* gene, particularly at codon 315 (*katG*315), are the most frequent followed by mutations in the *inhA* promoter region, especially at position -15 (*inhA*-15) (Seifert et al.,

2015; Vilchèze & Jacobs, 2014; Zhang & Yew, 2009). The presence of the *katG*315 mutation has been associated with high-level INH resistance and is considered one of the main causes of resistance in MDR-TB cases (Lu et al., 2014; Rintiswati & Praseno, 2016; Tilahun et al., 2020; van Soolingen et al., 2000). However, the frequency of this mutation can vary by geographic region (Bostanabad et al., 2008; Sadri et al., 2016; Suthum et al., 2020). Mutations in the *inhA* promoter region, encoding a key enzyme for cell wall synthesis, can lead to its overproduction. This overexpression allows bacteria to resist INH through a mechanism known as titration. Mutations in *inhA* confer low-level INH resistance as well as cross-resistance to ETO (Cao et al., 2023; Morlock et al., 2003a; Ranjan et al., 2023; Vadwai et al., 2013; Vilchèze & Jacobs, 2014). Double mutation at *inhA* and *katG* genes have been linked to increased minimum inhibitory concentration (Lempens et al., 2018), slower sputum culture conversion, and increased risk of fluoroquinolone resistance (Click et al., 2020).

2.10.2. RIF

RIF exhibits potent bactericidal activity against actively growing MTB (Maltempe et al., 2017). It also sterilizes slow-growing bacterial populations (Jindani et al., 2003; Mitchison, 1985; Palomino & Martin, 2014). RIF resistance is primarily liked to mutations in the rpoB gene, which encodes the β-subunit of ribonucleic acid (RNA) polymerase, a crucial enzyme for bacterial RNA synthesis. The binding of RIF to the β-subunit of ribonucleic acid (RNA) polymerase results in inhibition of transcription initiation (Dookie et al., 2018; Eddabra & Neffa, 2020; Xu et al., 2021; Zhang & Yew, 2015b). Mutations in the 81-bp region (codons 507-533) of the *rpoB* gene, also called rifampicin resistance determining region (RRDR), harbor over 95% of RIF resistance in MTB isolates and high-level RIF resistance is usually

associated with point mutations in 531, 526 and 516 codons (Hirani et al., 2020b; Jamieson et al., 2014). Predominant RIF resistance mutation outside RRDR has also been reported in Eswatini (Sanchez-Padilla et al., 2015). RIF resistance mutations often come with a fitness cost, hindering bacterial growth and transmission compared to wild-type strains (Gagneux et al., 2006; Knight et al., 2015). However, secondary mutations in the *rpoA* and *rpoC* genes have been identified as compensatory mechanisms that can mitigate this fitness cost (Conkle-Gutierrez et al., 2024; Emane et al., 2021; Merker et al., 2018). The majority of compensatory mutations in *rpoA/rpoC* are usually linked to the most prevalent clinical RIF resistance mutation *rpoB* S450L (Gygli et al., 2017).

2.10.3. EMB

EMB, a long-used drug for DS-TB treatment, has emerged as an add-on drug in the treatment of MDR and XDR-TB (Barliana et al., 2023; WHO, 2022d). It is a bacteriostatic drug that is active only against growing bacteria (Chauhan et al., 2021). Similar to PZA, resistance to EMB arises due to a wide range of genetic variants that can hinder its ability to disrupt arabinogalactan synthesis (Mabhula & Singh, 2019). The *embCAB* operon encodes arabinosyl transferases, which are involved in the biosynthesis of the mycobacterial cell wall, and mutations in this operon can disrupt the action of EMB, leading to resistance (Alcaide et al., 1997; Sun et al., 2017; Telenti et al., 1997; Xiang et al., 2021). Mutations among *embCAB* operon are responsible for around 70% clinical EMB resistant MTB (Xiang et al., 2021).The *embB* gene mutations, particularly at codon 306, are significantly associated with EMB resistance in MTB , and these mutations could potentially be used as molecular markers for the detection of MDR-TB (Hafeez et al., 2021; Munir et al., 2017).

2.10.4. PZA

PZA, a pro-drug, is a critical first-line drug for treating both DS-TB and MDR-TB strains susceptible to PZA. Its unique strength lies in its ability to sterilize dormant bacteria (persisters) even in acidic environments. This sterilizing effect allows for shortened treatment regimens, ultimately leading to a cure for TB (Werngren et al., 2017). The primary mechanism of PZA resistance in MTB is associated with mutations in the *pncA* gene, which encodes the enzyme pyrazinamidase (PZase) necessary for converting PZA into its active form, pyrazinoic acid (Rajendran & Palaniyandi, 2022; Wabale, 2016). Mutations in the *pncA* gene lead to a loss of PZase activity, thereby preventing the conversion of PZA to its bactericidal form and conferring resistance (Cheng et al., 2000; Khan et al., 2019; Scorpio et al., 1997; Tunstall et al., 2021). Mutations in the *pncA* gene exhibit a high degree of variability and occur throughout the entire gene sequence (Whitfield et al., 2015; Zhang & Yew, 2015b). Consequently, the development of reliable probe-based real-time PCR assays for detecting PZA resistance is still challenging (Tam et al., 2019).

2.10.5. FLQs

FLQs play a critical role in the treatment of MDR-TB. They are considered cornerstone drugs due to their bactericidal activity against MTB, favorable safety profile, and lack of cross-resistance with first-line anti-TB drugs (Ahmad & Mokaddas, 2011). Newer FLQs, such as LFX and MFX, are particularly important in MDR-TB regimens and are recommended by the WHO as part of the treatment (Shim, 2015; Shim & Jo, 2013; WHO, 2020, 2022d). These drugs target the DNA gyrase enzyme, essential for DNA replication

and cell division of MTB. The mechanism involves the stabilization of the gyrase-DNA complex, preventing the rebinding of cleaved DNA strands, interfering with DNA synthesis and ultimately leading to MTB cell death (Aubry et al., 2004; Kumar et al., 2021). Notably, mutations occurring in the quinolone-resistance-determining region (QRDR) of the *gyrA* and *gyrB* genes, which encode DNA gyrase, are the main cause of resistance to FLQs (Chen et al., 2012; Singh et al., 2022). These mutations, particularly those occurring at codons 90, 91, and 94 of *gyrA* QRDR predominate among MTB isolates (Avalos et al., 2015; Chakravorty et al., 2011; Singh et al., 2022).

2.10.6. SLIDs

Aminoglycosides like STM and AM are currently used as add-on therapies for DR-TB treatment, while SLIDs such as kanamycin (KAN) and capreomycin (CAP) are no longer prescribed due to concerns about their effectiveness and the emergence of resistance (Cegielski et al., 2021; Dillard et al., 2021; WHO, 2020). STM, once a mainstay TB treatment, is now reserved as a second-line option and is used in specific situations, such as when AM is unavailable, confirmed resistance to AM exists with STM susceptibility, or a fully oral treatment regimen cannot be formulated (WHO, 2020). It targets the bacterial ribosome, interfering with protein synthesis, and mutations in the *rrs* gene can alter the ribosome's structure, reducing the binding affinity of streptomycin and thus conferring resistance (Zhang & Young, 1994). Non-synonymous mutations in *rrs* and *rpsl* genes (encode 16 rRNA and S12 ribosomal protein, respectively) are associated with STM resistance in MTB isolates (Arjomandzadegan & Gravand, 2015; Finken et al., 1993; Jagielski et al., 2014). Mutations in the *gidB* gene, which encodes a 16S rRNA-specific methyltransferase, have also been implicated in STM resistance, although these mutations

confer a lower level of resistance compared to *rrs* and *rpsL* mutations (Jagielski et al., 2014; Okamoto et al., 2007).

The mechanism of drug resistance in MTBC for SLIDs (KAN, CAP and AM) is primarily associated with mutations in the *rrs* gene, which encodes the 16S rRNA. The A1401G mutation in the *rrs* gene is prevalent and has been linked to high-level resistance to these SLIDs (Du et al., 2013; Georghiou et al., 2012; Jugheli et al., 2009). Mutations in the *eis* promoter and *tlyA* gene have been implicated in resistance, particularly to CAP (Georghiou et al., 2012; Zhang et al., 2014).

2.10.7. ETO

ETO is a pro-drug that is a structural analog of INH. For ETO to be effective against MTB, it needs activation by *ethA* encoded mono-oxygenase enzyme, which is regulated by *ethR*. The activated drug targets the enzyme *inhA,* which is involved in cell wall biosynthesis (Brossier et al., 2011). The majority of mutations that lead to resistance to ETO have been identified in the *ethA* and *inhA* genes (Ushtanit et al., 2022). Mutations within these two genes were associated with relatively high levels of ETO resistance (Morlock et al., 2003b). Cross resistance between INH and ETO has also been reported, conferred by *inhA* mutation (Cao et al., 2023; Ranjan et al., 2023; Vilchèze & Jacobs, 2014).

2.10.8. New and repurposed drugs

The resistance mechanisms to new and repurposed TB drugs are becoming clearer through genomic studies, which is essential for advancing TB management. DLM and PA, both from the nitroimidazole class, are crucial in treating DR-TB. The primary mechanism of resistance involves mutations in the bioactivation pathway genes such as *ddn*, which

encodes deazaflavin-dependent nitroreductase (Ddn), and genes for the biosynthesis of the cofactor F420 (Lee et al., 2020; Nguyen et al., 2023). These mutations impair the activation of the drugs, reducing their effectiveness against MTB. Notably, some mutations confer resistance to one drug while maintaining susceptibility to the other, indicating that DLM and PA might not be fully interchangeable (Mansjö et al., 2022; Mudde et al., 2022). Although the emergence of resistance in clinical isolates remains low, continuous monitoring is essential for optimal treatment strategies (Liu et al., 2018).

BDQ and CFZ face resistance mechanisms involving both target-based and non-target-based mutations. BDQ, an ATP synthase inhibitor, primarily encounters resistance through rare mutations in the *atpE* gene. More commonly, resistance and cross-resistance to CFZ involve mutations in *Rv0678*, which regulates the MmpS5-MmpL5 efflux pump (Andries et al., 2014; Snobre et al., 2023). Efflux pump regulation mutations can decrease drug efficacy and are selected by sub-lethal concentrations of BDQ and CFZ (Andries et al., 2014). Additionally, mutations in *mmpR* and *pepQ* genes contribute to resistance, altering protein functionality and leading to decreased drug susceptibility (Shi et al., 2023).

In summary, the drug resistance mechanism of anti-TB drugs involves a complex interplay of genetic mutations and other cellular mechanisms that hinder the efficacy of current treatments. A comprehensive understanding of these mechanisms is crucial for developing rapid diagnostic tools, effective treatments, and novel drug targets to combat drug-resistant TB (Da Silva & Palomino, 2011; Singh et al., 2020).

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHODS

3.1. Study area

The study was conducted at two TB referral diagnostic laboratories found in Harar and Adama, Ethiopia which serve as referral centers for Mycobacteria culture and drug susceptibility testing (Figure 8).

The Adama TB regional laboratory is located in Adama city and serves nine zones in Oromia, as well as the surrounding Amhara and Afar regions. Adama, formerly known as Nazareth, is a large city in central Ethiopia located in rift valley about 100 km southwest of Addis Ababa. Adama serves as a major transportation hub, situated on the main highway and railway connecting Addis Ababa to Djibouti. It is located at longitude of 8.514477° N and latitude of 39.269257° E and elevation of 1712 meters above sea level (Temesgen & Hameed, 2015).

The Harar TB regional laboratory, located in Harar city 500 km from the capital, provides referral diagnostic services to Diredawa, east Harerge, west Harerge, Somali, and Harari regions. Harar is the capital city of the Harari region and is considered the fourth holiest city in Islam. The ancient city is located on a hilltop in the eastern part of the country and is about 500 kilometers from the Ethiopian capital Addis Ababa at an elevation of 1,885 meters above sea level. The city's coordinates are 9.3124° N, 42.1218° E. Harar was an important commercial center for centuries, linked by trade routes to the Arabian Peninsula, Asia, and the rest of the Horn of Africa. The old walled city of Harar Jugol was designated a UNESCO World Heritage Site in 2006 in recognition of its cultural significance (Girma et al., 2022).

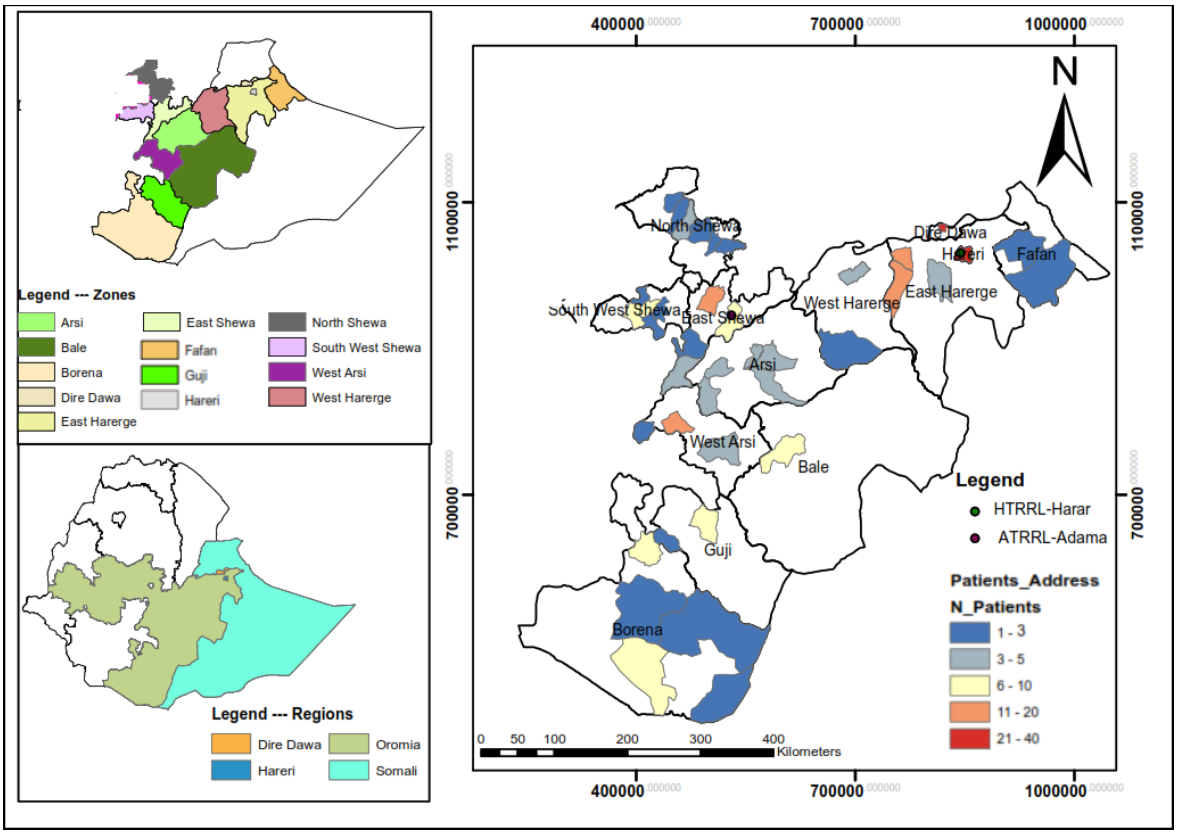

Figure 8: Regions, zones and addresses of study participants.

3.2. Study design and study isolates

This is a health facility-based cross-sectional study conducted at two TB referral diagnostic laboratories found in Harar and Adama, Ethiopia. From August 2018 to January 2019, 232 MTBC culture positive isolates were collected from pulmonary TB patients referred to Adama and Harar TB regional laboratories located in the premises of Adama Public Health Research and Referral Laboratory Center and Harar Health Research and Regional Laboratory, respectively. Demographic (age, sex, and address) and clinical (history of treatment) characteristics were collected.

3.3. Study categories

3.3.1. Study 1: Genetic diversity of MTB isolates

A total of 223 MTBC isolates which had successful spoligotyping results were used.

3.3.2. Study 2: Profile and frequency of drug resistance conferring mutations

A total 224 culture-positive MTBC isolates obtained from pulmonary TB patients on which GenoType MTBDRplus v2.0. and GenoType MTBDRsl v2.0. tests were performed and had valid results were included.

3.3.3. Study 3: Genomic characterization of MDR *Mycobacterium tuberculosis* isolates

For this study, a total of 79 MDR MTBC isolates with successful genotypic DST and genotyping result were included. Of the seventy-nine, 29 isolates from East Ethiopia were subjected to WGS analysis.

3.4. Culture and MTBC isolates

The sputum specimens were processed following standard procedures and inoculated onto the BACTEC™ MGIT™ 960 broth culture system (BD, Sparks, MD, USA). The growth of MTBC was confirmed by Capilia TB-Neo (Tauns laboratories, Japan) and AFB smear staining (Shen et al., 2009). The isolates confirmed as MTBC were then subcultured onto Lowenstein Jensen media and harvested within 3-4 weeks. Two cryo-vials containing 1ml 7H9 liquid medium were prepared and two loopful of colonies of MTBC culture were transferred to each vial which was later transported to the University of Pretoria's Medical Microbiology Department, TB laboratory for further testing.

3.5. DNA extraction

DNA extraction was performed by either heat inactivation at 80°C for 60 minutes in a water bath or using the PrimeXtract^{TM} kit (Longhorn Vaccines and Diagnostics, San Antonio, TX, USA). Briefly, 200µL of the culture together with 200µL of 100% ethanol and 200µL lysis buffer were transferred to a 1.5mL microcentrifuge and vortexed. The mixture was then centrifuged and incubated for 5 minutes at room temperature. The entire content was then transferred to an extraction column and centrifuged at 13,000rpm for 60 seconds. After removing the extraction column, the eluate was discarded. The extraction column was then filled with 500µL of wash buffer, centrifuged, and the eluate was removed. This step was repeated twice followed by washing of the filter with additional 500µL wash buffer and centrifugation of the extraction column to remove trace wash buffer. Finally, the nucleic acid was eluted by 1min of centrifugation at 13,000rpm using 50µL of preheated (60-70°C) elution solution. The extracted DNA was stored at -20°C for further use.

3.6. Genotyping

The isolates were genotyped using spoligotyping following the procedure described by Kamerbeek et al., 1997 and following kit supplier's instructions (Ocimum Biosolutions Company, IJsselstein, The Netherlands). Briefly, the set of spacers in the isolates was amplified by PCR using DRa and DRb primers. The amplicons were then hybridized on a reference set of 43 spacers impregnated on a membrane (Animal and Plant Health Agency, Great Britain). The presence or absence of spacers was visualized on a film (Hyperfilm ECL, Amersham) as black and white squares, which were later converted to

binary codes (1/0) for analysis. Known strains of *M. bovis* Bacillus Calmette–Guérin (BCG) and *M. tuberculosis* H37Rv were utilized as positive controls, and Qiagen water (Qiagen Company, Germany) was used as a negative control.

3.6.1. Spoligotype assignment and database comparison

The spoligotype patterns entered into the MS Excel spreadsheet were converted into binary and octal formats using the SITVITWEB website (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/tools.jsp#) which were later compared with online international databases such as SITVIT2 (Couvin et al., 2019b), SpolLineages (Couvin et al., 2020) and RUN TB-lineage (Aminian et al., 2010, 2014). Sub-lineages and International Shared Types (SIT) were obtained using the updated version of SPOLDB4 and SITVITWEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/batch.jsp). Conformal Bayesian Network (CBN) major lineages, and SNP-based lineages were determined using online tools RUN TB-lineage (https://tbinsight.cs.rpi.edu/run_tb_lineage.html) and SpolLineages (http://www.pasteur-guadeloupe.fr:8081/SpolLineages/spol.jsp), respectively. CBN does not distinguish between the West African (L6 and L5) and Ethiopian (L7) lineages, classifying them as *Mycobacterium africanum*. We used SpolLineages to avoid this, further verify the CBN classification of major lineages and explore the classification of lineages as generalists and specialists (Coll et al., 2014; Stucki et al., 2016).

A dendrogram was constructed based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm using the MIRU-VNTRplus identification database to determine the molecular clustering of the isolates. The UPGMA tree was

then retouched using FigTree v1.4.4. Two or more isolates with similar spoligotype patterns were defined as a cluster. A spoligotype pattern that was not previously reported in an international database was defined as orphan or new whereas a spoligotype with SIT reported once in the study was defined as unique.

Geographic mapping of MTBC lineage and sub-lineages was performed using QGIS v3.22.6. The shape files of study sites from where the isolates collected were obtained from UNOCHA website (https://data.humdata.org/dataset/cod-ab-eth).

3.7. Genotypic Drug Susceptibility testing

Susceptibility testing to RIF and INH as well as to FLQs and SLIDs was performed using GenoType MTBDR*plus* v2.0 and GenoType MTBDR*sl* v2.0, respectively (Hain Lifescience, Nehren, Germany). All the procedures that included master mix preparation, amplification and hybridization were performed following the manufacture's instruction (HAIN Lifescience, 2015a, 2015b).

3.7.1. Interpretation of results

The evaluation sheets provided with the kits were used to paste the developed strips and determine the resistance status according to the manufacturer's instruction. Four control zones on each strip ensure the test proceeds smoothly and the reagents perform well. These include the conjugate control (CC) that demonstrates the efficiency of the conjugate binding and substrate reaction, the amplification control (AC) excludes mistakes during extraction and amplification and the carry-over of amplification inhibitors, MTBC control (TUB) hybridizes with amplicons derived from all members

of the MTBC and the locus controls (*rpoB, katG, and inhA* (MTBDRplus) *gyrA, gyrB, rrs* and *eis* (MTBDRsl)) detect a gene region specific for the respective locus.

In each strip, there are Wild Type (WT) probes and Mutant (MUT) probes corresponding to each gene studied. When at least one of the WT bands is missing and the corresponding MUT band appeared, it indicated the presence of known mutation in the gene of the tested strain suggesting drug resistance. Additionally, the absence of WT band without the corresponding MUT band was interpreted as resistance due to unknown mutations. The presence of all WT bands and the absence of all MUT bands was interpreted as an isolate susceptible to the drugs tested. The presence of all WT bands together with MUT bands was defined as heteroresistant (HAIN Lifescience, 2015a, 2015b).

3.8. Whole genome sequencing

WGS sequencing was performed at Longhorn Vaccines and Diagnostics (San Antonio, Texas, USA) on 29 MTB isolates collected from east Ethiopia. The Nextera XT Sample Prep Kit (Illumina, San Diego, CA, USA) was used to process the clinical isolate DNA (1-5 ng) and the Nextera XT 135 Index Kit (Illumina, San Diego, CA, USA) was used to pool the DNA. Libraries were assigned into sets of 24 and pooled following the manufacturer's instructions (Illumina, San Diego, CA, USA). NGS was carried out according to the manufacturer's instructions (Illumina, San Diego, CA, USA) utilizing the MiSeq 600 cycle reagent kit (V3). SeqMan NGen (V8) and LaserGene (V13) Core Suite (DNAStar Inc., Madison, WI, USA) were used for bioinformatic analysis including contig assembly, multiple sequence alignments and mutation analysis. Trimming of

adaptors and artifacts plus removal of poor sequences with low base quality scores was achieved by trimmomatric software. Furthermore, FASTQ files were uploaded to web-based bioinformatics pipelines Exatype software (Hyrax Biosciences, Cape Town, South Africa) to predict DR; TB-profiler v4.4 (Coll et al., 2015; Phelan et al., 2019) and Mykrobe (Hunt et al., 2019) for DR prediction and lineage/sub-lineage assignment.

3.8.1. Cluster and genomic relatedness analysis based on core-genome multi-locus sequence typing (cgMLST)

The default settings and functionalities of the Ridom SeqSphere+ software (Pipeline Mode) version 9.0.10 (Ridom GmbH, Mendelstr, 11 D-48149 Münster, Germany), were employed to import raw FASTQ files, assemble the genomes and perform cgMLST analysis. Briefly, the raw FASTQ files were uploaded, aligned to a reference of genome MTB strain H37Rv (GenBank accession no. NC_00962.3). Finally, the Ridom SeqSphere+ MTB cgMLST scheme with 2891 core genes was employed for cluster analysis and creation of minimum spanning tree by ignoring pairwise missing values. The software identified successful alignments to cgMLST as good targets and performed full analysis on isolate sequences with >95.0% of such targets. Cluster distance threshold of ≤12 alleles and ≤5 alleles were used for cluster/genomic related analysis and to define a strain as part of a recent transmission event, respectively (Bouzouita et al., 2019; Kohl et al., 2014, 2018).

3.9. Quality control

For each run of MTBDR*plus* and MTBDR*sl* assays, molecular grade water and reference strain H37Rv susceptible to all drugs tested were used as negative and positive controls, respectively. For spoligotyping, known strains of *M. bovis* Bacillus Calmette–Guérin (BCG) and *M. tuberculosis* H37Rv were utilized as positive controls, and Qiagen water (Qiagen Company, Germany) as a negative control.

3.10. Statistical Analysis

Data were first entered into Excel spreadsheet; cleared, and analyzed using the SPSS statistical software package, V20 (SPSS Inc., Chicago, IL, USA). Frequencies and percentages were used to describe clinical and sociodemographic characteristics, linesges/sublineges as well as drug resistance conferring mutations. Tables and figures were used to present the results. A logistic regression model was used to assess variables associated with clustering in terms of the odds ratio and 95% confidence interval (CI). The chi-squared test was applied to compare categorical data. A p-value less than or equal to 0.05 was considered significant.

3.11. Ethical Considerations

The study was ethically approved by College of Natural and Computational Sciences' Institutional Review Board (IRB), Addis Ababa University and permission to transfer isolates to South Africa was obtained from the Ethiopian Food, Medicine and Health Care Administration and Control Authority (now known as the Ethiopian Food and Drug Administration) and the Health Department of South Africa. The study utilized isolates routinely obtained from patients for diagnostic and therapeutic purposes. No personal information of patients was collected.

CHAPTER 4

RESULTS

CHAPTER 4: RESULTS

4.1. Genetic diversity of *Mycobacterium tuberculosis* isolates from the central, eastern and southeastern Ethiopia

4.1.1. Characteristics of the study population

Of the 232 isolates collected, 9 were either lost or did not have successful spoligotyping result. Hence, this study examined a total of 223 isolates from TB patients who were presumed to have DR-TB, with a mean age of 30.4 years. Most of the study subjects (58.3%) were males and in the age group-15-34 years (58.3%). The majority (80.8%) were from the Central (Arsi; East, North, Southeast and West Shewa) and Eastern (Diredawa, Harar, Jigjiga, East and West Hararge) parts of Ethiopia. The remaining were from the Southeastern part of Ethiopia, specifically from Bale, Borena, Guji, West Arsi and West Guji zones. There was an equal proportion of patients in relation to treatment history (Table 2).

Table 2: Characteristics of study subjects

Variable		Frequency (Percent)
Age, years		
	≤ 15	19 (8.5)
	16-24	56 (25.1)
	25-34	74(33.2)
	35-44	38(17.0)
	>45	34(15.2)
	Missing	2(.9)
Sex		
	Male	130 (58.3)
	Female	93 (41.7)
History of anti-TB drug treatment		
	New	112 (50.2)
	Previously treated	111 (49.8)
Region		
	Central	94 (42.2)
	Southeastern	43 (19.3)
	Eastern	86 (38.6)

4.1.2. Genetic diversity of *Mycobacterium tuberculosis* lineages/sub-lineages

Spoligotyping was used to genotype 223 isolates, and 77 different spoligotype patterns were identified, of which 42 spoligotype patterns comprising 186 isolates were registered in the SITVIT2 database. Of these, 27 were unique while 15 were clustered with 2-49 isolates that accounted for 85.5% (159/186) of all isolates with known SIT. The remaining 35 orphan patterns, representing 16.6% (37/223) of the total isolates, were not found in the SITVIT2 database (Table 3, Table 4). The overall clustering rate of sub-lineages with known SIT was 71.3%. The proportion of clustered isolates was higher in each geographic region: Central (72.3%), Eastern (72.1%) and Southeastern (67.4%). There were five dominant spoligotypes with SIT, which accounted for over half of the

genotyped isolates 117 (52.5%): SIT149/T3_ETH (n = 49), SIT53/T1 (n = 33), SIT21/ CAS1_Kili (n = 24) and SIT41/Turkey (n = 11) (Figure 9). T and CAS were the dominant families, accounting for 48.9% and 16.6% of the isolates, respectively. Of the 49 isolates with SIT149/T3_ETH sub-lineage, 8 (16.3%) were children of 15 years or younger. According to the CBN analysis, 94.6% of the total 223 isolates belonged to two major lineages: EA/L4 (75.3%) and EAI/L3 (19.3%). The remaining 12/223 (5.4%) isolates were represented by EAS/L2, IO/L1, ETH/L7 and MA (L5 and L6) which were represented by five, three, one and three isolates, respectively. Of those classified by CBN as EA and EAI, 8.3% (EA) and 4.7% (EAI) were not known by SNP-based lineage analysis of the SpolLineages online tool. Additionally, we found two generalist sub-lineages (Coll F. et al., 2014; Stucki et al., 2016), L4.1.2/Haarlem (n=5) and L4.3/LAM (n=5), and most (9/10) were isolated from the central and eastern regions (Table 3).

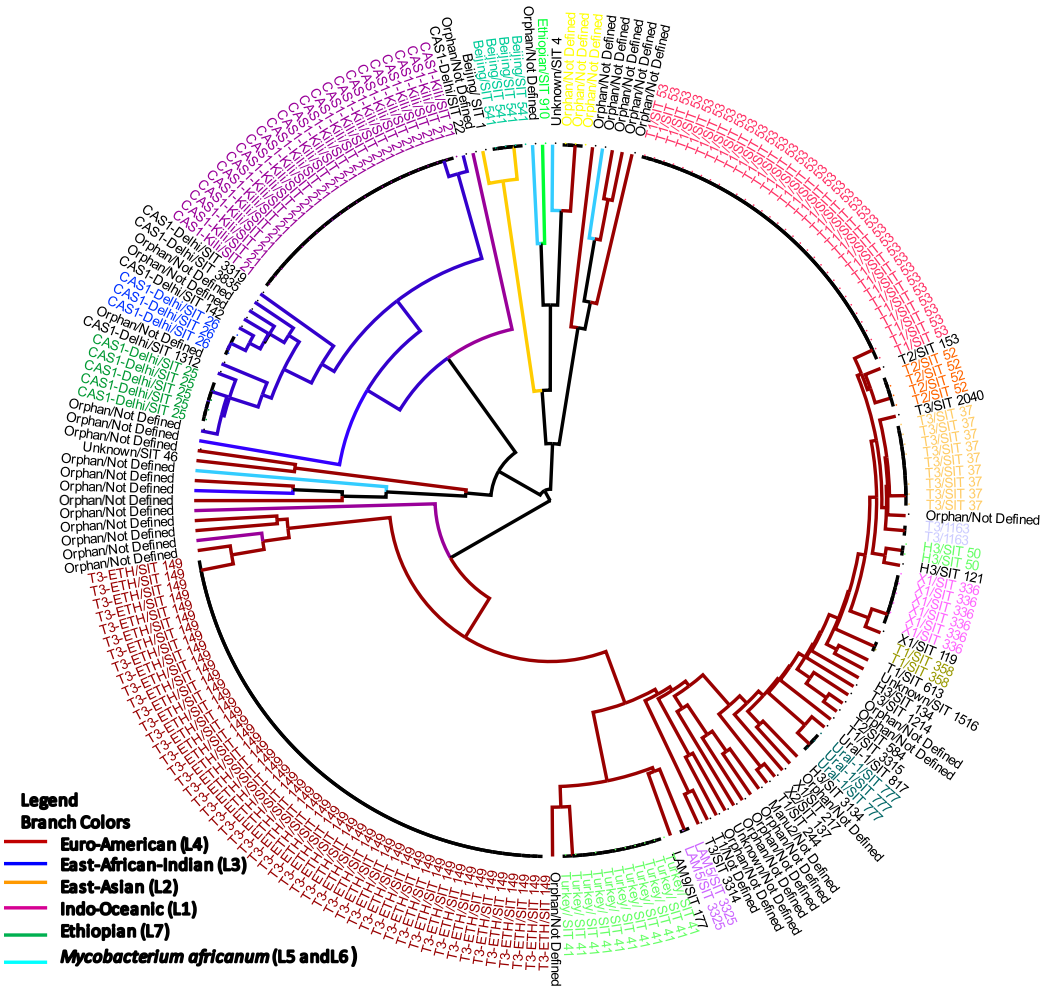

Figure 9: Radial UPGMA tree based on spoligotyping data of 223 MTB isolates from Central, Eastern and Southeastern parts of Ethiopia.

Annotations with similar colored fonts in the outer ring represent clustered sub-lineages/spoligotypes. The ones written in black fonts are unique/orphan isolates. The colors of branches in the inner circle indicate the major lineages.

Table 3: Description of 35 orphan strains (n = 37) and the corresponding spoligotyping defined lineages/sub-lineages recorded among MTB strains isolated from Central, Eastern and Southeastern Ethiopia.

Octal code	Binary code	SITVIT2	CBN	SNP-based lineage
525252525240521	■□■□■□■□■□■□■□■□■□■□■□■□■□■□■□■□■□■□■	ND	**EA**	EA (L4.1.1)
007777777760771	□□□□□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	**EA**	EA (L4)
500177740003171	■□■□□□□□□□■■■■■■■■■■□□□□□□□□□□□□■■□□■■■■■	ND	**EAI**	EAI (L3)
501252500002121	■□■□□□□□■□■□■□■□■□■□□□□□□□□□□□□□■□□□■□■□■	ND	**EAI**	EAI (L3)
777000377777771	■■■■■■■■■□□□□□□□□□□■■■■■■■■■■■■■■■■■■■■■	ND	**IO**	UNK
700006037177661	■■■□□□□□□□□□□□□■■□□□□□□■■■■■□□■■■■■■■■■□■■□■	ND	**MA**	WA (L5 and L6)
703757740003171	■■■□□□□■■■■■■□■■■■■■■■□□□□□□□□□□□□□■■□□■■■■■	ND	**EAI**	EAI (L3)
713247664001661	■■■□□■□■■□■□■□□■■■■■□■■□■□□□□□□□□□□■■■□■■□■	ND	**IO**	UNK
777777777000171	■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□■■■■■	UKN	**EA**	UNK
771046024100261	■■■■■■□□■□□□■□□■■□□□□□■□■□□□□■□□□□□□□■□■■□■	ND	**EA**	UNK
773777776000771	■■■■■■□■■■■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■	ND	**EA**	UNK
774242525042000	■■■■■■■□□□■□■□□□■□■□■□■□■□■□□□■□□□■□□□□□□□□	ND	**MA**	UNK
703357740003071	■■■□□□□■■□■■■□■■■■■■■■□□□□□□□□□□□□□■■□□□■■■■	ND	**EAI**	EAI (L3)
703777710003771	■■■□□□□■■■■■■■■■■■■■■□□■□□□□□□□□□□■■■■■■■■■	ND	**EAI**	UNK
777737777360771	■■■■■■■■■■■■□■■■■■■■■■■■■■□■■■■□□□□■■■■■■■	ND	**EA**	EA (L4)
777775477760731	■■■■■■■■■■■■■■■■□■■□□■■■■■■■■■■■□□□□■■■□■■■	ND	**EA**	EA (L4)
777777404060771	■■■■■■■■■■■■■■■■■■■□□□□□■□□□□□■■□□□□■■■■■■■	ND	**EA**	EA (L4.3)
477777376413771	■□□■■■■■■■■■■■■■■□■■■■■■■□■□□□□■□■■■■■■■■■	ND	**IO**	IO (L1)
777737377750771	■■■■■■■■■■■■□■■■■■■□■■■■■■■■■■■■□■□□□■■■■■■■	ND	**EA**	UNK
510047236343661	■□■□□■□□□□□□■□□■■■■□■□□■■■■□□■■■□□□■■■■□■■□■	ND	**MA**	WA (L5 and L6)
525252500000000	■□■□■□■□■□■□■□■□■□■□■□□□□□□□□□□□□□□□□□□□□□	ND	**EA**	UNK
551246236340261	■□■■□■□□□■□■□■□□■■□□■□□□■■■■□□■■■□□□□□□■□■■□■	ND	**EA**	EA (L4)
511047236340261	■□■□□■□□□■□□□■□□■■■■□■□□■■■■□□■■■□□□□□□■□■■□■	ND	**EA**	EA (L4)
777347777763771	■■■■■■■■■□■■■□□■■■■■■■■■■■■■■■■■□□■■■■■■■■■	Manu2	**EA**	UNK
511046637561671	■□■□□□■□□□■□□□■□□■■□■■□□■■■■■■□■■■□□□□■■■□■■■■	ND	**EA**	UNK
603777700003771	■■□□□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□□■■■■■■■■■	ND	**EAI**	UNK
311000377760771	□■■□□■□□□■□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	ND	**EA**	EA (L4)
777000377730771	■■■■■■■■■□□□□□□□□□□■■■■■■■■■■■■□■■□□□■■■■■■■	ND	**EA**	UNK
713357776363771	■■■□□■□■■□■■■□■■■■■■■■■■■■■□□■■■■□□■■■■■■■■■	ND	**EA**	UNK
777347636361771	■■■■■■■■■□■■■□□■■■■■■□□■■■■■□□■■■■□□□■■■■■■■■	ND	**EA**	UNK
777775777760721	■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■□■□■□■	ND	**EA**	EA (L4)
774177770000021	■■■■■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□□□□□■□■	ND	**EA**	UNK
520000377760771	■□■□■□□□□□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	ND	**EA**	EA (L4)
700000017760771*	■■■□□□□□□□□□□□□□□□□□□□□■■■■■■■■■□□□□■■■■■■■	ND	**EA**	EA (L4)
777357677761771	■■■■■■■■■□■■■□■■■■■■□■■■■■■■■■■■□□□■■■■■■■■	ND	**EA**	UNK

EA=Euro-American, EA=East-Asian, EAI=East-African-Indian, IO=Indo-Oceanic, UKN=Unknown, WA=West Africa, MA= *Mycobacterium Africanum*, ND=Not defined,**n=3*

Table 4: Description of 42 shared types (SITs; n = 186 isolates) registered in the SITVIT2 or SpolDB4 database and the corresponding spoligotyping defined lineages/sub-lineages isolated from the Central, Eastern and Southeastern Ethiopia.

Octal code	Binary code	SITVIT2	CBN	SNP-based lineage	SIT	Number of isolates
777000377760771	■■■■■■■■■□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	T3-ETH	EA	EA (L4)	149	49
777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	EA	EA (L4)	53	33
703377400001771	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	CAS1-Kili	EAI	EAI (L3)	21	24
777777404760771	■■■■■■■■■■■■■■■■■■■■□□□□□■□□■■■■■□□□□■■■■■■■	Turkey	EA	EA (L4.2.2.1)	41	11
777737777760771	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T3	EA	EA (L4)	37	9
777776777760731	■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■□■■■	X1	EA	EA (L4.1.1)	336	6
703777740003171	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□■■□□■■■■■	CAS1-Delhi	EAI	EAI (L3)	25	5
777777777760731	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	EA	EA (L4)	52	4
000000000003711	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□□■■	Beijing	EAS	EAS (L2)	541	4
703777740003771	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	CAS1-Delhi	EAI	EAI (L3)	26	3
777777777420771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	Ural-1	EA	EA (L4.2.1)	777	3
677737777760771	■■□■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T3	EA	EA (L4)	1163	2
776737407760771	■■■■■■■■□■■■□■■■■■■□□□□□■■■■■■■□□□□■■■■■■■	LAM5	EA	EA (L4.3)	3325	2
717777777760771	■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	EA	EA (L4)	358	2
777777777720771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	H3	EA	EA (L4.1.2)	50	2
000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing	EAS	EAS (L2)	1	1
777776777760771	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	X1	EA	EA (L4.1.1)	119	1
777777775720771	■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■□■□□□□■■■■■■■	H3	EA	EA (L4.1.2)	121	1
777617777760771	■■■■■■■■■■■□□□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T3	EA	EA (L4)	1214	1
703777740003131	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□■■□□■□■■■	CAS1-Delhi	EAI	EAI (L3)	1312	1
777777777720631	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■□□■■■	H3	EA	EA (L4.1.2)	134	1
777776777760601	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■□□□□■	X2	EA	EA (L4.1.1)	137	1
703777700003771	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	CAS1-Delhi	EAI	EAI (L3)	142	1
777377777761771	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■	UKN	EA	UNK	1516	1
757777777760731	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	EA	EA (L4)	153	1
377777607760771	□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	LAM9	EA	EA (L4.3)	177	1
777737777760371	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□□■■■■■■	T3	EA	EA (L4)	2040	1
777736777760771	■■■■■■■■■■■■□■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	X1	EA	EA (L4.1.1)	217	1
703777400001771	■■■□□□□■■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	CAS1_Dehi	EAI	EAI (L3)	22	1
777777777760601	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	T1	EA	EA (L4)	244	1
777737377720771	■■■■■■■■■■■■□■■■■■□■■■■■■■■■■□■□□□□■■■■■■■	H3	EA	EA (L4.1.2)	3134	1
177000377760771	□□■■■■■■■□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	T3-ETH	EA	EA (L4)	3141	1
006737777760771	□□□□□□■■□■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T3	EA	EA (L4)	3314	1
376777737760771	□■■■■■■■□■■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	T1	EA	EA (L4)	3315	1
703417740003771	■■■□□□□■■■□□□□■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	CAS1-Delhi	EAI	EAI (L3)	3319	1
703177740003771	■■■□□□□■■□□■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	CAS1-Delhi	EAI	EAI (L3)	3835	1
000000007760771	□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	UKN	EA	EA (L4.3)	4	1
777777770000000	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□	UKN	EA	UKN	46	1
777775777760731	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	EA	EA (L4)	584	1
777177777760771	■■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	EA	EA (L4)	613	1
777777777420731	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■□■■■	Ural-1	EA	EA (L4.2.1)	817	1
700000007177771	■■■□□□□□□□□□□□□□□□□□□□□□□□■■■□□■■■■■■■■■■■■■	ETH	MA	ETH (L7)	910	1

EA=Euro-American, EA=East-Asian, EAI=East-African-Indian, IO=Indo-Oceanic, ETH=Ethiopian, UKN=Unknown, WA=West Africa, MA= *Mycobacterium Africanum*, ND=Not-defined

4.1.3. Geographical distribution of the lineages/sub-lineages

EA (L4) contributed a significantly high proportion to the lineage distribution across the study sites: 83.7% (Southeastern), 81.9% (Central) and 64.0% (Eastern) of the isolates collected in the respective parts of Ethiopia (Appendix 1). EAI (L3) was reported mainly in the eastern regions, followed by the central regions, with proportions of 62.8% and 27.9% of the total isolates, respectively. With the exception of Guji/West Guji, where no EAI was recorded, EA and EAI were identified in all zones from where the isolates were acquired. MTBC isolates obtained from Arsi (n=2) and Guji (n=1) zones were identified as *M. africanum* (Figure 10, Appendix 2). The SIT910 spoligotype (ETH/L7), which is confined to Ethiopia, was isolated from West Arsi. There were five isolates with Beijing sub-lineage (EAS/L2), four from the eastern (three from Diredawa and one from Harar) and one from the central part (Southwest Shewa) of Ethiopia. Of note, 10/11 (90.9%) of EA (L4) lineage, SIT41 spoligotype/Turkey sub-lineage were reported from southeastern Ethiopia (Borena and Guji). On the contrary, the SIT149 spoligotype/T3-ETH sub-lineage and SIT53 spoligotype/T1 sub-lineages were reported from most of the sites where the isolates were collected (Figure 11, Appendices 3 and 4).

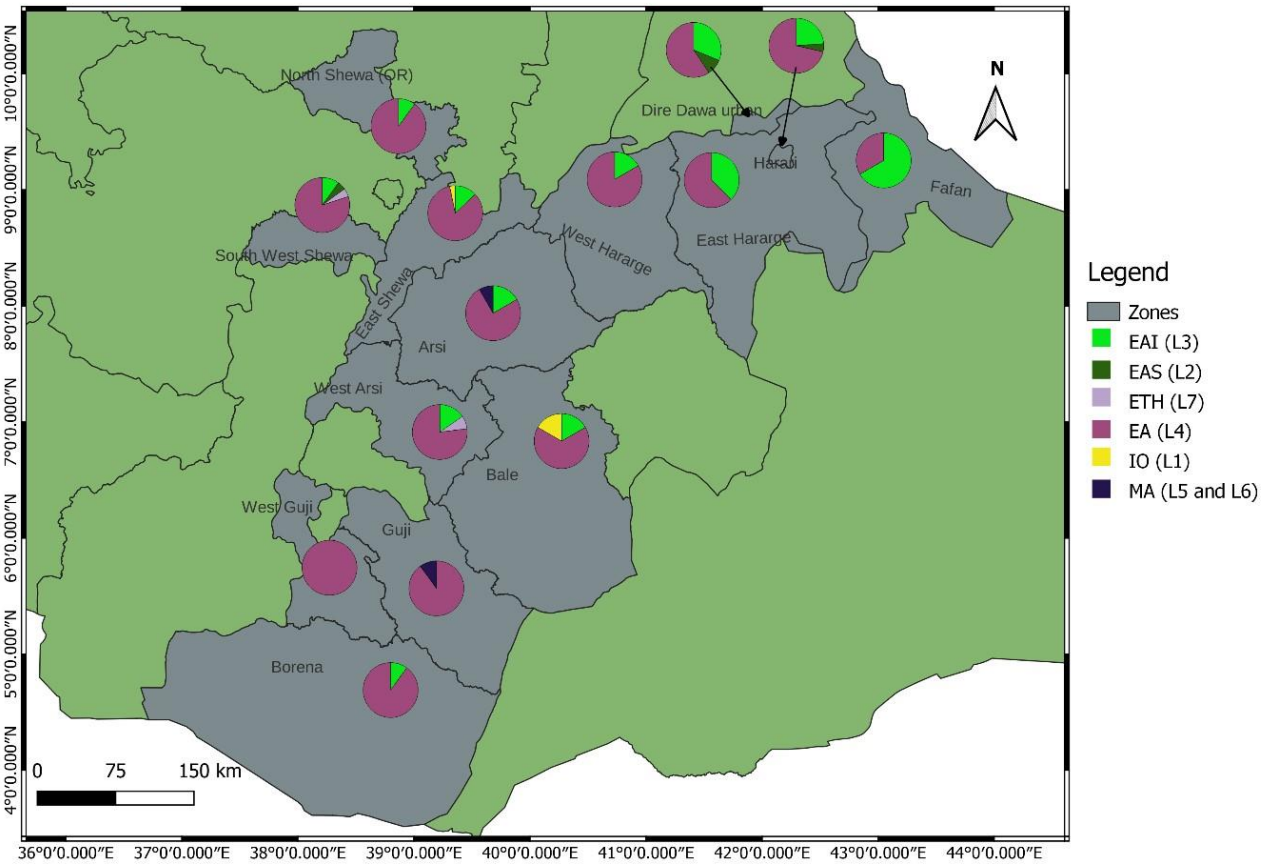

Figure 10: Distribution of major lineages in Central, Eastern and Southeastern Ethiopia. Pie charts show the proportions of the six lineages among MTBC isolates in each zone from where isolates were obtained. The size of the circle does not correspond to the number of isolates analyzed. The actual numbers are shown in Appendix 2: color codes are as in Figure 10. A total of 223 MTBC isolates were included.

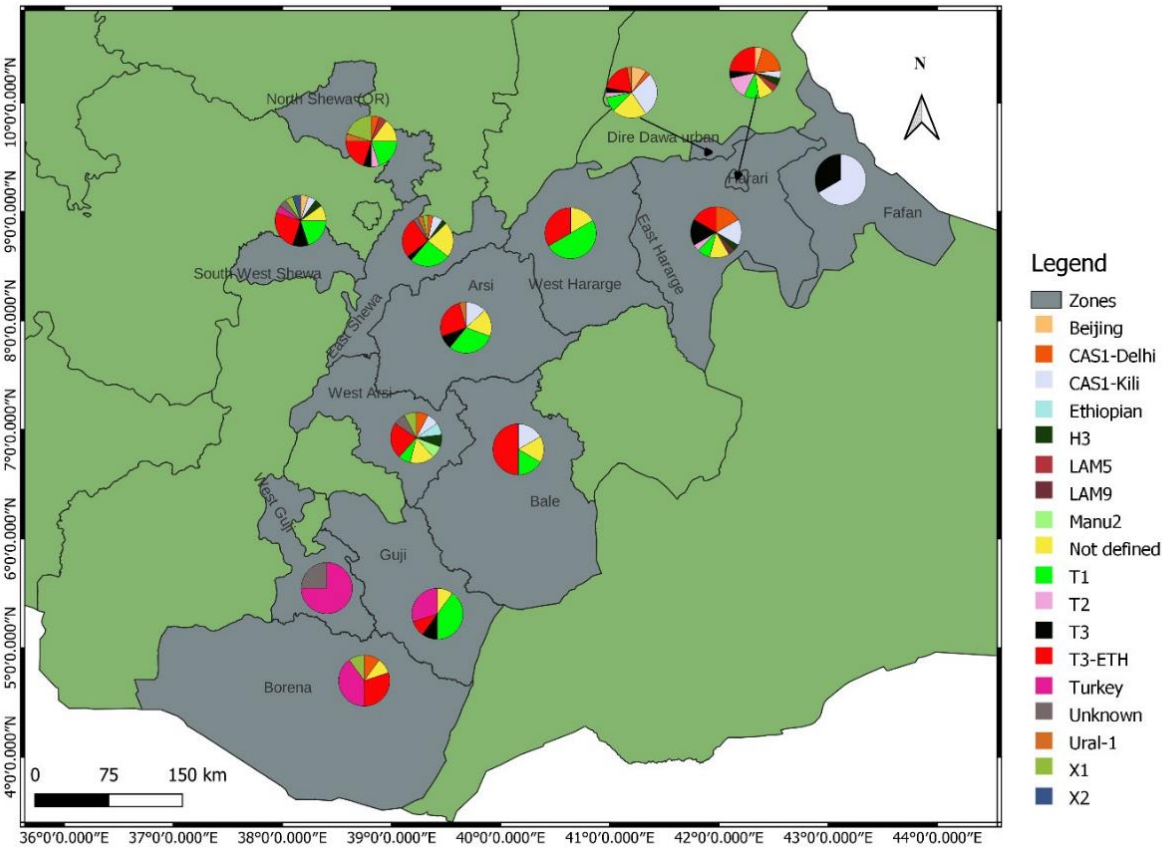

Figure 11: Distribution of MTBC sub-lineages in Central, Eastern and Southeastern Ethiopia.

Pie charts show the proportions of the sub-lineages among MTBC isolates in each zone from where isolates were obtained. The size of the circle does not correspond to the number of isolates analyzed. The actual numbers are shown in Appendix 4: color codes are as shown in Figure *11*. A total of 223 MTBC isolates were included.

4.1.4. Comparison of factors associated with clustering

In a bivariate logistic regression analysis, factors such as age, treatment history, and lineage were statistically associated with isolate clustering at p-values 0.2. However, there was no statistically significant association (p-value>0.05) of sex and region with clustering. Age group was the only variable statistically associated with clustering of MTBC isolates (p-value<0.05) in multivariable analysis (Table 5).

Table 5: Comparison of patient characteristics with clustering of MTBC isolates from the central, eastern and southeastern Ethiopia.

Variable	Category	Clustered (N/%)	Unique (N/%)	COR (95% CI)	P-value	AOR (95% CI)	P-value
Age	≤ 15	17 (89.5)	4 (10.5)	2.975 (0.823-10.760)	0.096	3.055 (0.831-11.230)	0.093
	16-24	36 (64.3)	20 (35.7)	1.260 (0.525-3.022)	0.605	1.393 (0.572-3.390)	0.465
	25-34	61 (82.4)	13 (17.6)	3.285 (1.324-8.146)	0.01	3.728 (1.467-9.470)	0.006
	35-44	25 (65.8)	13 (34.2)	1.346 (0.517-3.505)	0.543	1.363 (0.517-3.593)	0.531
	≥ 45	20 (58.8)	14 (41.2)	1		1	
Sex	Male	95 (73.1)	35 (26.9)	1.230 (0.685-2.209)	0.488		
	Female	64 (68.8)	29 (31.2)	1			
Region	Central	68 (72.3)	26 (27.9)	1.012 (0.527-1.945)	0.97		
	Southeast	29 (67.4)	14 (32.6)	0.802 (0.363-1.772)	0.585		
	East	62 (72.1)	24 (27.9)	1			
Treatment	New	74 (66.7)	37 (33.3)	0.635 (0.354-1.141)	0.129	0.644 (0.350-1.185)	0.158
	Previously treated	85 (75.9)	27 (24.1)	1		1	
Lineage	EA (L4)	123 (73.2)	45 (26.8)	1.443 (0.751-2.770)	0.201	1.842 (0.919-3.689)	0.061
	Others	36 (65.5)	19 (34.5)	1			

4.2. Profile and frequency of mutations conferring drug-resistant tuberculosis in the central, southeastern and eastern Ethiopia

4.2.1. Characteristics of study population

In the current study, a total of 224 culture positive isolates were included. The median age of the patients from which isolates were collected was 28 years (±SD=12.19, range 9-69 years) and the majority (n=132, 58.9%) were males. The majority (n=181, 80.8%) of the patients were from the central (Arsi, East/North and South West Shoa zones) and eastern (Diredawa, Jigjiga, Harar, East and West Hararge zones) regions of Ethiopia.

4.2.2. Drug susceptibility testing

4.2.2.1. GenoType MTBDRplus

Of 224 isolates, 88 (39.3%) and 85 (38.0%) were resistant to RIF and INH, respectively. Of those resistant to RIF, 82 (93.2%) were also resistant to INH and 6 (6.8%) were monoresistant. More than a third of the isolates (n=82, 36.6%) were MDR-TB. The majority (n=66, 80.5%) of them were from previously treated, males (n=51, 62.2%) and those aged between 15-34 years (n=50, 60.9%) patients. Furthermore, 37 (45.1%) of those isolates identified as MDR-TB were from the eastern Ethiopia followed by the central Ethiopia (n=26, 31.7%). There were 3 (1.3%) INH monoresistant isolates (Figure 12, Table 6).

4.2.2.2. GenoType MTBDRsl

The MTBDR*sl* v2.0. was performed on 73 MDR-TB and 6 RR isolates. Of these, 77 (MDR=71, RR=6) had an interpretable result and were included in the current study.

Further resistance to FLQs and SLIDs was observed only in MDR-TB isolates. Accordingly, 7 (9.1%) were resistant to FLQs and 3 (3.9%) were resistant to SLIDs. All of the FLQs resistant isolates were from previously treated patients with first-line anti-TB drugs. However, 2 (66.7%) of the SLIDs resistant isolates were from new MDR-TB patients. Furthermore, all of the SLIDs and 4 (57.1%) of the FLQ resistant isolates were from the eastern Ethiopia (Figure 12, Table 6).

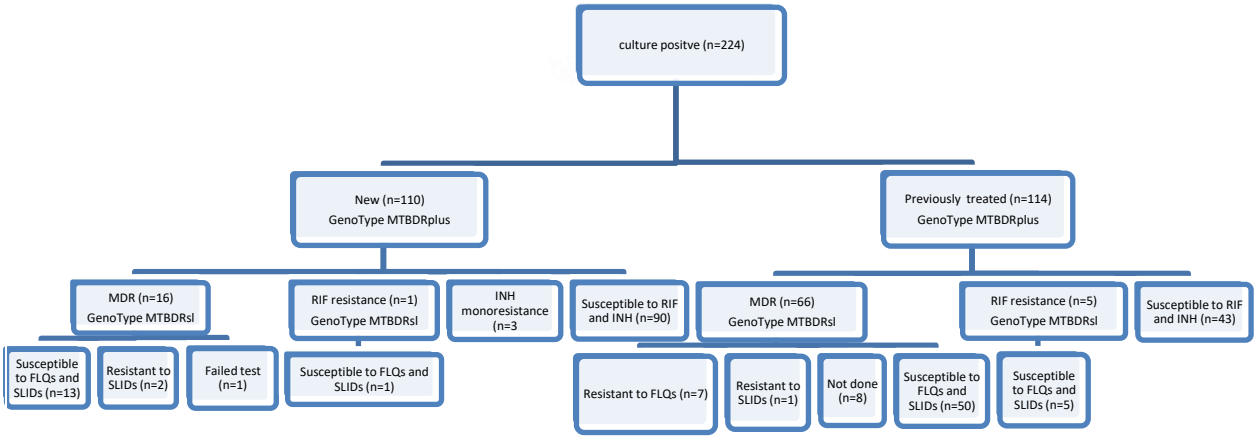

Figure 12: Study population and study flow diagram.

Table 6: Comparison of patient characteristics and drug resistance profile

Variable		Any INH resistance (N=85) [N (%)]		Any RIF resistance (N=88) [N (%)]		MDR (N=82) [N (%)]		FLQs resistance (N=7) [N (%)]		SLIDs resistance (N=3) [N (%)]	
		PreRXed	New	PreRXed	New	PreRXed	New	PreRXed	New	PreRXed	New
Sex	Male	44 (51.8)	9 (10.6)	49 (55.7)	8 (9.1)	44 (53.7)	7 (8.5)	4 (57.1)	0	1 (33.3)	0
	Female	22 (25.9)	10 (11.8)	22 (25.0)	9 (10.2)	22 (26.8)	9 (11.0)	3 (42.9)	0	0	2 (66.7)
Age (years)	<15	7 (8.2)	1 (1.2)	7 (8.0)	1 (1.1)	7 (8.5)	1 (1.2)	0	0	0	0
	15-24	12 (14.1)	9 (10.6)	13 (14.8)	8 (9.1)	12 (14.6)	8 (9.6)	0	0	0	1 (33.3)
	25-34	27 (31.8)	3 (3.5)	28 (31.8)	4 (4.6)	27 (32.9)	3 (3.7)	3 (42.9)	0	1 (33.3)	0
	35-44	9 (10.6)	4 (4.7)	9 (10.2)	3 (3.4)	9 (11.0)	3 (3.7)	2 (28.6)	0	0	0
	45-54	7 (8.2)	1 (1.2)	9 (10.2)	0	7 (8.5)	0	2 (28.6)	0	0	0
	≥55	4 (4.7)	1 (1.2)	5 (5.7)	1 (1.1)	4 (4.9)	1 (1.2)	0	0	0	1 (33.3)
Region	Central*	24 (28.2)	4 (4.7)	26 (29.5)	2 (2.2)	24 (29.3)	2 (2.4)	3 (42.9)	0	0	0
	Southeastern**	18 (21.2)	1 (1.2)	18 (20.5)	1 (1.1)	18 (21.9)	1 (1.2)	1 (14.3)	0	0	0
	Eastern***	24 (28.2)	14 (16.5)	27 (30.7)	14 (15.9)	24 (29.3)	13 (15.9)	3 (42.9)	0	1 (33.3)	2 (66.7)

PreRxed, previously treated; INH, isoniazid; RIF, rifampicin; FLQs, fluoroquinolones; SLIDs, Second-line injectable drugs; *Arsi, East/North and South West Shoa zones; ** Diredawa, Jigjiga, Harar, East and West Hararge zones; ***Bale, Borena, Guji and West Arsi zones

4.2.3. Frequency of mutations conferring drug resistance to INH, RIF, FLQs and SLIDs

4.2.3.1. Mutations in the rpoB

The majority of isolates (n=52, 59.1%) with RIF resistance had mutation at codon S531L followed by D516V (n=13, 14.8%), H26Y (n=8, 9.1%) and H526D (n=3, 3.4%). The remaining (13.6%) had missing WT band without the corresponding MUT band and were reported as unknown mutations. These included WT7 mutation (n=8, 9.1%) and WT8 mutation (n=4, 4.5%) (Table 7).

Table 7: Mutations conferring drug resistance to RIF and INH

Drug	Gene	Failing wild type band	Developing mutation band	Mutation	MDR (N=82) [N (%)]	RR (N=6) [N (%)]
Rifampicin	*rpoB*	WT3, WT4	MUT1	D516V	13 (15.9)	0
		WT7	MUT2B	H526D	3 (3.7)	0
		WT7	MUT2A	H526Y	7 (8.5)	1 (16.7)
		WT7		Unknown	6 (7.3)	2 (33.3)
		WT8		Unknown	4 (4.9)	0
		WT8	MUT3	S531L	49 (59.8)	3 (50.0)
					MDR (Total N=82)	**INH MNR (Total N=3)**
					N (%)	**N (%)**
Isoniazid	*katG*	WT	MUT1	S315T1	78 (95.1)	0
		WT	MUT2	S315T2	4 (4.9)	0
	inhA	WT1	MUT1	c-15t	0	3 (100.0)

*RIF, Rifampicin; INH, Isoniazid; MNR, Monoresistant; MDR, Multidrug resistant; RR, Rifampicin resistant

4.2.3.2. Mutations in the katG and inhA

From 85 isolates with INH resistance, the majority (n=82, 96.5%) of the isolates had mutation at codon 315 of the *katG* gene (n=78, 91.8% S315T1 and n=4, 4.7% S315T2) indicating high level resistance. Mutation in the promoter region of *inhA* gene (which indicate low level resistance) was observed in the INH monoresistant isolates (n=3, 100%) collected from drug naïve patients. All had mutation at codon -15 (C-15t). No isolate had a co-mutation at *katG* and *inhA* genes (Table 7).

4.2.3.3. Mutations in the gyrA and rrs

A total of 7 isolates (9.1%) showed mutations conferring drug resistance to FLQs. The *gyrA* mutation A90V was observed in 3 (42.9%) isolates, whereas mutations D94A, D94G, D94N/Y were each observed in 1 isolate (14.3%). One isolate was heteroresistant where it had all the WT probe together with MUT probe (A90V and D94N/Y) (Table 8).

No *gyrB* mutations were observed in this study. Additionally, 3 MDR-TB isolates had missing WT band and without the corresponding MUT band at the *rrs* gene.

Table 8: Mutations conferring drug resistance to FLQs and SLIDs

Drug	Gene	Failing wild type band	Developing mutation band	Mutation	MDR (N=73) [N (%)]	RR (N=6) [N (%)]
Fluoroquinolones	*gyrA*	WT2	MUT1	A90V	3 (4.1)	0
		WT3	MUT3A	D94A	1(1.4)	0
		WT3	MUT3C	D94G	1(1.4)	0
		WT3	MUT3B	D94N, D94Y	1(1.4)	0
		All present	MUT1, MUT3B	A90V, D94N, D94Y	1(1.4) *	0
Second-line injectable drugs	*rrs*	WT		Unknown	3 (4.1)	0

*Heteroresistant

4.3. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolates in central, eastern and southeastern Ethiopia.

4.3.1. Study population

Seventy-nine MDR-TB isolates confirmed by GenoType MTBDRplus v2 and selected from 232 MTB isolates. MTB isolates confirmed as MDR but had no fingerprinting results were excluded. Seven of the MDR MTBC isolates had additional resistance to FLQs based on GenoType MTBDR*sl* and were defined as pre-XDR TB (Viney et al., 2021). Less than one-fifth (n=15,19.0%) of the isolates were obtained from patients with no history of previous TB treatment. The majority (n=49, 62.0%) of isolates were from male patients, while the remaining (n=30, 38.0%) were from female patients, with a mean age of 28 years (range, 12–60 years). A slightly higher proportion (n=34, 43.0%)

of isolates were from eastern (Diredawa, Harar, East and West Hararge) Ethiopia, followed by central Ethiopia (n=26, 32.9%), and southeast Ethiopia (n=19, 24.1%) (Table 9).

Table 9: General characteristics of patients from which isolates were collected.

Characteristics	**N (%)**
Sex	
Male	49 (62.0)
Female	30 (38.0)
Age	
<25	28 (35.4)
25-45	41 (51.9)
>45	10 (12.7)
Previous history of TB treatment	
Previously treated	64 (81.0)
Not previously treated	15 (19.0)
Genotypic Resistance	
INH+RIF[§]	69 (87.3)
INH+RIF+SLIDs[§]	3 (3.8)
INH+RIF+FLQs[§§]	6 (7.6)
INH+RIF+FLQs+SLIDs[§§]	1 (1.3)
Address	
*Central	26 (32.9)
**East	34 (43.0)
***Southeast	19 (24.1)

*Arsi, East, North and Southwest Shewa; **Diredawa, Harar, East and West Hararge; ***Bale, Borena, Guji and West Arsi, [§]MDR; [§§]pre-XDR

4.3.2. Genetic diversity of MDR MTBC strains based on spoligotyping

Genotyping of 79 MDR MTB isolates by spoligotyping resulted in 25 different spoligotype patterns, 16 patterns containing 70 (88.6%) isolates were registered in the international database and the remaining 9 (11.4%) were orphan or new. Five spoligotype patterns consisting of 59 isolates, genetic diversity of 31.7%, were clustered with 4 to 26 isolates per cluster (Figure 13). Assessment of lineage distribution showed EA/L4 (n=56, 70.9%) as most encountered, followed by EAI/ L3 (n=16, 20.3%). Others included EAS/L2 (n=4, 5.1%), ETH/L7 (n=1, 1.3%), IO/L1 (n=1, 1.3%) and MA (n=1, 1.3%). Further classification into sub-lineages showed the predominance of T3-ETH, SIT149 (n=26, 32.9%) and CAS1_Kili, SIT21 (n=14, 17.7%) followed by T1, SIT53 (n=9, 11.4%), Turkey, SIT41 (n=6, 7.6%) and Beijing, SIT541 (n=4, 5.1%). The remaining (n=20, 25.3%) were unique isolates with SITs (n=11, 13.9%) and orphan/new isolates (n=9, 11.4%). Overall, T (48.1%) and CAS (20.3%) families were dominant among the MDR MTBC isolates (Table 10).

Table 10: Frequency of spoligotype patterns and assigned lineages/sub-lineages of 79 MDR *Mycobacterium tuberculosis* isolates in central, eastern and southeastern Ethiopia

SIT	Binary code	Octal code	SITVIT2	CBN	Number of isolates (%)
149	■■■■■■■■□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	777000377760771	T3-ETH	EA	26 (32.9)
21	■■■□□□□■■□■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	703377400001771	CAS1-Kili	EAI	14 (17.7)
53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	T1	EA	9 (11.4)
41	■■■■■■■■■■■■■■■■■■■□□□□□■□□■■■■■□□□□■■■■■■■	777777404760771	Turkey	EA	6 (7.6)
541	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□□■■	000000000003711	Beijing	EAS	4 (5.1)
4	□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	000000007760771	UKN	EA	1 (1.3)
22	■■■□□□□■■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	703777400001771	CAS1_Delhi	EAI	1 (1.3)
26	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	703777740003771	CAS1-Delhi	EAI	1 (1.3)
50	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	777777777720771	H3	EA	1 (1.3)
52	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	777777777760731	T2	EA	1 (1.3)
336	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■□■■■	777776777760731	X1	EA	1 (1.3)
358	■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	717777777760771	T1	EA	1 (1.3)
910	■■■□□□□□□□□□□□□□□□□□□□□□□□■■■□□■■■■■■■■■■■■■	700000007177771	ETH#	MA	1 (1.3)
1516	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■	777377777761771	UKN	EA	1 (1.3)
3315	□■■■■■■■□■■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	376777737760771	T1	EA	1 (1.3)
3325	■■■■■■■■□■■■■□■■■■■■■□□□□□■■■■■■■■□□□□■■■■■■■	776737407760771	LAM5	EA	1 (1.3)
Orphan or New	■□■□■□□□□□□□□□□□□□□■■■■■■■■■■■■■■□□□□■■■■■■■	520000377760771	ND	EA	1 (1.3)
Orphan or New	□■■□□■□□■□□□□□□□□□□■■■■■■■■■■■■■■□□□□■■■■■■■	311000377760771	ND	**EA**	1 (1.3)
Orphan or New	■■■■■■■□□□■□■□□□■□■□■□■□■□■□■□□□■□□□■□□□□□□□□	774242525042000	ND	**MA***	1 (1.3)
Orphan or New	■■■■■■■■■■■■□■■■■■■■■■■■■■□■■■■□□□□■■■■■■■	777737777360771	ND	**EA**	1 (1.3)
Orphan or New	■■■■■■■■■■■■■■■■□■■□□■■■■■■■■■■■□□□□■■■□■■■	777775477760371	ND	**EA**	1 (1.3)
Orphan or New	■□□■■■■■■■■■■■■■■□■■■■■■■■□■□□□□■□■■■■■■■■■	477777376413771	ND	**IO****	1 (1.3)
Orphan or New	■■■■■■■■■□■■■■□□■■■■■■■■■■■■■■■□□■■■■■■■■■	777347777763771	Manu2	**EA**	1 (1.3)
Orphan or New	■□■□□■□□■□□□■□□□■□□■■□■■□□■■■■■■■□■■■□□□■■■□■■■■	511046637561671	ND	**EA**	1 (1.3)
Orphan or New	■■■□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■□□□□■■■■■■■	700000017760771	ND	**EA**	1 (1.3)

ND=Not defined, UKN=Unknown; * Assela; **Bishoftu; #Shashemene

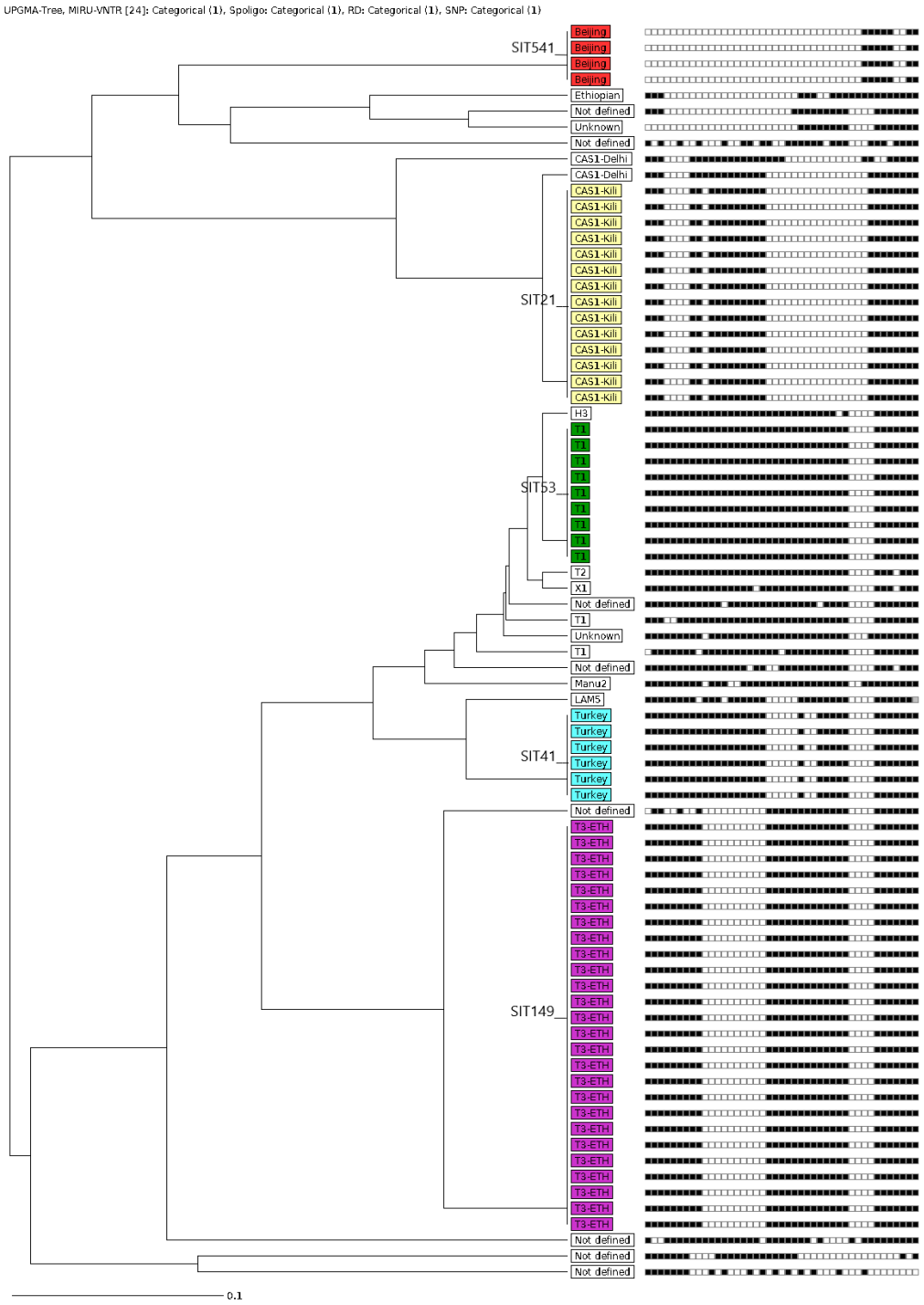

Figure 13: Spoligotyping based phylogenetic tree of 79 MDR MTB isolates in central, eastern and southeastern Ethiopia.

Sub-lineage (study number) and spoligotype pattern are indicated. Clustered isolates are shaded in color.

4.3.3. Whole Genome Sequencing

Of the 29 randomly selected MTBC isolates on which WGS was performed, isolates with low read coverage (n=2), pansusceptible (n=2) and RIF monoresistant (n=3) were excluded from analysis. Therefore, 22 isolates with at least mutations conferring drug resistance to RIF and INH were included.

4.3.3.1. Drug resistance patterns, mutations and MTBC lineages

Among the 22 isolates analyzed, WGS identified that 11 (50%) of the MDR MTB isolates belonged to EA/L4, 7 (31.8%) to EAI/L3 and 4 (18.2%) to EAS/L2. Within EA/L4, 10 isolates were classified as L4.2.2, with one isolate falling under the L4.8 sub-lineage. All EAI/L3 and EAS/L2 isolates were attributed to L3.3.1 and L2.2.1 sub-lineages, respectively. None of the isolates exhibited resistance to drugs such as amikacin and new/repurposed drugs, yet 17 (77.3%) displayed additional drug resistance to one or more anti-TB drugs. Predominant drug resistance was observed against EMB (n=14, 63.6%), STM (n=11, 50%) and PZA (n=7, 31.8%), with relatively lower proportion resistance to FLQs (n=3, 13.6%), ETO (n=3, 13.6%) and CAP (n=1, 4.6%) (Table 11, Appendix 5).

Table 11: Proportion of drug resistance and lineages of 22 MDR MTB isolates in East Ethiopia

			N	%
Lineage	Lineage 2 (East Asian)	L2.2.1	4	18.2
	Lineage 3 (East African Indian)	L3.3.1	7	31.8
	Lineage 4 (Euro American)	L4.2.2	10	45.5
		L4.8	1	4.5
Resistance	MDR	RIF+INH	5	22.7.
		RIF+INH+ other drugs*	14	63.6
	Pre-XDR	RIF+INH+ other drugs+ FLQs	3	13.6

*Any of the following: EMB, PZA, STM, ETO, CAP

Among the RIF resistant isolates, a prevalent mutation at codon 450 of the *rpoB* gene was identified in more than half of the cases (n=12, 54.6%). Specifically, 10 isolates harbored the *rpoB*_S450L mutation, while one isolate displayed both *rpoB*_S450L and a compensatory *rpoC* mutation at codon G332R, and another exhibited the *rpoB*_S450C mutation. Additionally, mutations at codon 435 were observed in four isolates (18.2%) and included D435V mutation, whereas mutations at codon 445 were detected in five isolates, consisting of H445C (n=2, 9.1%), H445G (n=2, 9.1%), and H445D (n=1, 4.5%). A double mutation at codons H445Y and T427P was present in one isolate (4.5%).

Importantly, the occurrence of the *rpoB* mutation at codon 435 was exclusive to the L2.2.1 Beijing strains in this study. *katG*_S315T mutation was responsible for INH resistance for all the isolates.

The EMB-resistant isolates (n=14, 63.6%) exhibited mutations in the *embA* and *embB* genes. These mutations included *embB*_M306V (n=5, 35.7%), *embB*_M306I (n=2, 14.3%), *embB*_H1002R (n=2, 14.3%), and *embA*_c.-12C>T (n=1, 7.1%). Double mutations were observed at codons M306I and G406S (n=2, 14.3%), as well as M306I and G406A (n=1, 7.1%) of the *embB* gene. Additionally, one isolate (7.1%) exhibited a double mutation at *embA*_c.-16C>T and *embB*_M306I.

Among the PZA-resistant isolates (n=7), five distinct mutations were identified in the *pncA* gene. These mutations comprised *pncA*_T135I (n=2, 28.6%), *pncA*_L27P (n=2, 28.6%), *pncA*_T135P (n=1, 14.3%), *pncA*_V139G (n=1, 14.3%), and *pncA*_192_193insA (n=1, 14.3%).

For the FLQs-resistant isolates (n=3), mutations in *gyrA* gene were observed, including D94G (n=1, 33.3%), D94N (n=1, 33.3%), and a double mutation at codons A90V and D94A (n=1, 33.3%). Of the 12 STM-resistant isolates, 2 had mutations in *rrs*, 2 in *rpsl,* and 5 in *gid* genes. Three STM mutants had double mutations in the *gid* and *rrs* genes. All ETO resistant strains had mutations in the *ethA* gene. The only strain with CAP resistance had mutation in the *tlyA* gene (Table 12, Appendix 5).

Table 12: Summary of mutations conferring drug resistance to first- and second-line anti-TB drugs among 22 MDR *Mycobacterium tuberculosis* isolates in East Ethiopia.

Drug (N)	**Mutation***	**MDR (N)**	**Pre-XDR (N)**	**Total (N/%)**
RIF (22)	*rpoB*_S450L	9	1	10/45.5
	*rpoB*_D435V	4	-	4/18.2
	*rpoB*_H445C	1	1	2/9.1
	*rpoB*_H445G	1	1	2/9.1
	*rpoB*_S450L; *rpoC*_G332R	1	-	1/4.5
	*rpoB*_S450C	1	-	1/4.5
	*rpoB*_H445D	1	-	1/4.5
	*rpoB*_H445Y + *rpoB*_T427P	1	-	1/4.5
INH (22)	*katG*_S315T	19	3	22/100.0
EMB (14)	*embB*_M306V	4	1	5/35.7
	*embB*_M306I	2	-	2/14.3
	*embB*_M306I; *embB*_G406S	1	1	2/14.3
	*embB*_H1002R	2	-	2/14.3
	*embB*_M306I*; embB*_G406A	1	-	1/7.1
	*embA*_c.-12C>T	1	-	1/7.1
	*embA*_c.-16C>T; *embB* _306I	-	1	1/7.1
PZA (7)	*pncA*_T135I	2	-	2/28.6
	*pncA*_L27P	1	1	2/28.6
	*pncA*_T135P	1	-	1/14.3
	*pncA*_V139G	1	-	1/14.3
	*pncA*_192_193insA	1	-	1/4.3
STM (11)	*gid*_c.102_103insG	3	-	3/27.3
	*rrs*_A514C; *gid*_c.386_386del	2	1	3/27.3
	*gid*_c.386_386del	1	-	1/9.1
	*rrs*_A514C	1	-	1/9.1
	*rrs*_C517T	1	-	1/9.1
	*rpsL*_K43R	1	-	1/9.1
	*rpsL*_K88R	1	-	1/9.1
ETO (4)	*ethA_c.-11A>G*	1	1	2/40.0
	*ethA*_M1R	-	2	1/40.0
	ethA_c:g.4326474_4326615del	1	-	1/20.0
FLQs (3)	*gyrA*_A90V; *gyrA*_D94A	-	1	1/33.3
	*gyrA*_D94G	1	-	1/33.3
	*gyrA*_D94N	-	1	1/33.3
CAP (1)	*tlyA*_c.358_359insG	-	1	1/100.0

*For RIF mutations, add 81 to find the corresponding *E. coli* numbering commonly used in GenoType MTBDR*plus/sl* reporting

4.3.3.2. Cluster analysis using cgMLST to decipher recent transmission of MDR-TB

Of the 22 isolates, 15 achieved the good target percentage of 95% and were thus included in the final cgMLST analysis. A significant proportion (46.7%) of the isolates were clustered (clustering rate=26.7%) into three different cgMLST clusters consisting three isolates of L2.2.1, two isolates of L4.2.2 and two isolates of L3.1.1. The remaining 8 isolates (53.3%) were unique, falling out of the upper limit of the cluster threshold of a maximum of 12 allele differences. Recent MDR-TB transmission events (a threshold of ≤5 allele variants) were recorded in each of the three clusters: cluster 1(BF26-BF60), cluster 2 (BF1-BF16) and cluster 3 (BF12-BF58). Cluster 1 belonged to Beijing sub-lineage L2.2.1. This cluster was characterized by resistance to only RIF and INH as well as a fixed rpoB_D435V (not detected in other strains) and katG_S315T mutations. Cluster 2 belonged to Euro-American sub-lineage L4.2.2 and was characterized by rpoB_S450L, katG_S315T and gid_c.102_103insG shared mutations. Cluster 3 which belonged to Delhi CAS/ East African Indian sub-lineage L3.1.1 also shared common mutation combination rpoB-H445G, katG-S315T, pncA-L27P, *embB*_M306I, *mbB* G406S, *rrs*-A514C, *gid*_c.386delG and *ethA*_c.-11A>G (Figure 14, Appendix 6).

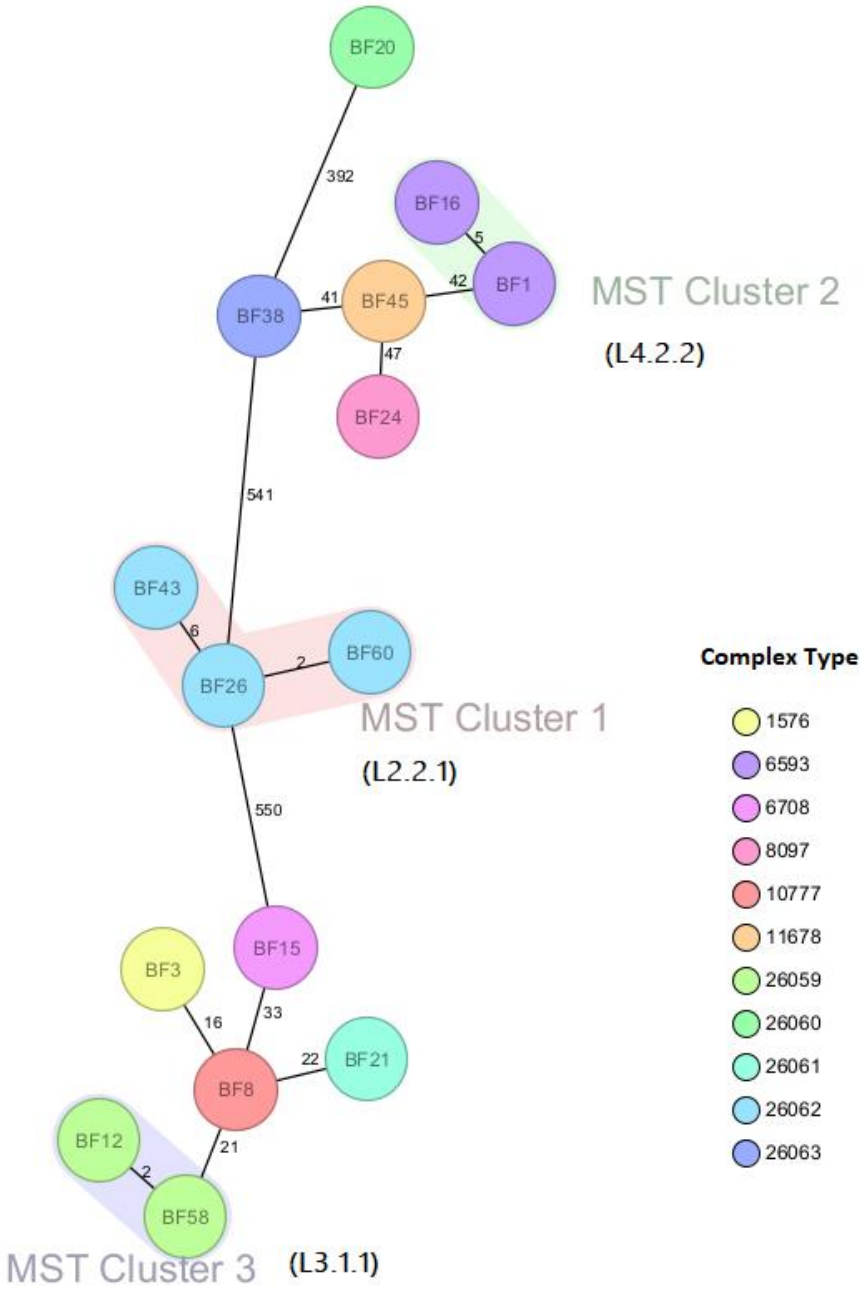

Figure 14: Ridom MSeqSphere+ minimum spanning tree (MST) for 15 MDR MTBC isolates from East Ethiopia based on 2891 columns.

*P*airwise ignoring missing values. MST Cluster distance threshold=12 alleles.

CHAPTER 5

DISCUSSION

CHAPTER 5: DISCUSSION

This section presents the findings from the three studies in the order they are discussed in Chapter IV. Following this, the limitations, conclusions, and recommendations for the whole study will be addressed.

5.1. Genetic diversity of MTBC isolates

A review on molecular epidemiology of MTBC in Ethiopia (Tulu & Ameni, 2018) highlighted the scarcity of data from certain regions such as Harari and pastoralist communities of Oromia which include the Borena and Guji zones. The low number of isolates identified from these regions and other less investigated areas would restrict the scientific community's ability to observe the full picture of the transmission dynamics and population structure of MTBC in Ethiopia. In line with this, the current study examined the genomic diversity of MTBC isolates obtained from patients in a large geographical area spanning from the central part of Ethiopia to the far Southeast (Moyale) bordering the northern part of Kenya and the East (Diredawa, Harar, and Jigjiga) doorway to Djibouti and Somalia.

Supporting previous studies summarized in two recent reviews (Abebe et al., 2019; Tessema et al., 2013), a heterogeneous MTBC population structure dominated by the T and CAS families with the corresponding EA (L4) and EAI (L3) lineages was shown in the current study. Three-quarters (75.3%) of the isolates in this study were members of EA (L4). A similar or higher proportion of EA (L4) has been reported from different parts of Ethiopia, including the current study sites (Abebe et al., 2019; Bedewi et al., 2017; Haile et al., 2020a; Lobie et al., 2020; Mekonnen et al., 2018; Merid et al., 2021;

Welekidan et al., 2021). However, a lower proportion (40.1%) of EA (L4) has been reported in northwest Ethiopia (Ejo et al., 2021). We also identified the globally predominant sub-lineages L4.1.2/Haarlem (n=5) and L.4.3/LAM (n=5). L4.1.2/Haarlem was previously shown to be connected to a high rate of transmission clusters (Tafess et al., 2021) in Ethiopia. It is also the third major sub-lineage in Ethiopia (Mekonnen et al., 2019; Tulu & Ameni, 2018), albeit isolated in modest proportions in the current study.

Various studies conducted in different parts of Ethiopia reported an overall low prevalence of IO (L1) and ETH (L7) (Ejo et al., 2021; Tafess et al., 2021). Accordingly, the current study identified three IO (L1) isolates from Bale, East, and Southwest Shewa and one ETH (L7) isolate from West Arsi. ETH (L7), first reported from Woldia (Firdessa et al., 2013), is predominant in the northern highlands of Ethiopia (Comas et al., 2015; Nebenzahl-Guimaraes et al., 2016). It has also been identified in different parts of Ethiopia (Ameni et al., 2013; Ayalew et al., 2021; Mengistu et al., 2015; Merid et al., 2021; Mihret et al., 2012; Wondale et al., 2020). Notably, this lineage is known to progress toward disease at a slower rate than other lineages (Yimer et al., 2015) and is mainly limited to Ethiopia and Ethiopian immigrants (Blouin et al., 2012; Coscolla & Gagneux, 2014). Consequently, extensive investigation is required to comprehend why it is unique to Ethiopia and Ethiopians.

Three isolates of the West African-bound MA (L5 and L6) lineages were also identified from Arsi and Guji. When verified using the SNP-based lineage identification tool (Couvin et al., 2020), one isolate was identified as unknown, whereas the other two were confirmed as MA (L5 and L6). There is an evidence that the MA (L5 and L6) are less virulent and are being replaced by EA (L4) in West Africa (Comas et al., 2015).

Conversely, in Ethiopia where EA (L4) is predominant, MA (L5 and L6) lineages have been isolated sporadically (Abebe et al., 2019; Bedewi et al., 2017; Haile et al., 2020b; Nuru et al., 2015; Wondale et al., 2020). Therefore, it is crucial to carefully examine the impact of these lineages on the epidemiology of TB in Ethiopia, as this may have implications for TB control strategies and treatment approaches.

Consistent with other investigations carried out in Ethiopia (Ameni et al., 2013; Garedew et al., 2013; Mihret et al., 2012; Zewdie et al., 2016), the T3-ETH sub-lineage/SIT149 spoligotype, reported to be more likely in a cluster (Tadesse et al., 2017; Tessema et al., 2013; Yimer et al., 2015), was the most frequent in our study, followed by the T1 sub-lineage/SIT53 spoligotype. A study conducted in Oromia region (Hussien et al., 2022), which included the zones from which two-thirds of our isolates were obtained, found a high proportion of clustered ST149 spoligotype followed by orphan spoligotypes rather than the commonly reported T or CAS sub-lineages. This could be attributed to several factors including differences in sampling techniques, the broad geographic area and high population of the region and the relatively small sample size used in the current study.

The high proportion of clustered spoligotypes, particularly SIT149, identified in different parts of the country magnifies its key role in TB transmission dynamics and disease burden in Ethiopia. Furthermore, the finding that 16.3% of the 49 isolates with spoligotype SIT149 in the current study were from children aged ≤15 years may indicate an ongoing TB transmission from adults to children. A similar proportion (16.6%) of the total isolates were not registered in the online SITVIT2/SPOLDB4 database suggesting that there is a need further clarify the population structure of MTBC causing TB in Ethiopia.

A relatively large cluster (n=11) of the SIT41 spoligotype, which is phylogeographically specific to Turkey (Kisa et al., 2012), was reported in this study with the majority (10/11) being from patients in southeastern (Borena and Guji) Ethiopia. This sub-lineage, although in small numbers (n≤5), was previously reported (Agonafir et al., 2010; Bedewi et al., 2017; Diriba et al., 2020; Ejo et al., 2021; Firdessa et al., 2013; Garedew et al., 2013; Hussien et al., 2022; Taye et al., 2021; Wondale et al., 2020) in different parts of Ethiopia. Similarly, it was reported to be less common in the African continent (Chihota et al., 2018). Clusters of larger size (n=6) from Guji and Liben area pastoralists (Gumi et al., 2012) and most recently from Arsi zone (n=7) (Tafess et al., 2021) were reported. The findings of our study strengthen the increasing importance of this clade in the pastoralist communities of the southeastern Ethiopia and call for further investigation to assess its role in TB disease burden of these regions and to implement appropriate interventions.

The widely studied Beijing sub-lineage of the EAS/L2 characterized by its worldwide geographical distribution and virulent properties (Coscolla & Gagneux, 2014) has previously been documented in some parts of Ethiopia (Ali et al., 2016; Biadglegne et al., 2015; Lobie et al., 2020; Taye et al., 2021). In this study, in addition to the classical Beijing strain-SIT1, we identified a rare spoligotype cluster, SIT541, characterized by the absence of two more spacers (40 and 41) compared to the predominant SIT1. At the time of our analysis, only 16 isolates were reported worldwide, representing 0.1% of the total registered Beijing lineage, as per the SITVIT2 database. Interestingly, in the present study, all SIT541 isolates were detected in eastern Ethiopia, specifically in Diredawa (three isolates) and Harar (one isolate). The exclusive detection of this spoligotype in

eastern Ethiopia and its limited global distribution may suggest a localized emergence and transmission of this strain in the region. Furthermore, the findings of the current study, together with a previous study reporting a similar frequency of SIT1 from Diredawa (Mekonnen et al., 2018), may indicate a unique epidemiological pattern and stress the evolving nature of the Beijing strain in the region, highlighting the need for enhanced surveillance and monitoring. A large-scale longitudinal study is required to better understand the pressure that this lineage might put on the community in the region and on the overall TB control effort of the region.

A high rate (71.3%) of clustered sub-lineages with known SIT was recorded in the current study. Besides, the proportion of clustered isolates was higher in each geographic region: Central (72.3%), Eastern (72.1%) and Southeastern (67.4%). A comparable rate of clustering has been reported from different regions across Ethiopia (Ameni et al., 2013; Bedewi et al., 2017; Belay et al., 2014; Maru et al., 2015; Zewdie et al., 2016). The high rate of clustering rate, although not with epidemiological link information, could indicate an ongoing transmission of TB in the respective regions. Variables, such as sex, geographic region, treatment history, and lineage were not significantly associated with an isolate being in a cluster. However, patients aged 25-34 years (AOR= 3.728, 95% CI (1.467-9.470)) were more likely to be part of a cluster compared to those aged >45 years. This may indicate that individuals in this age group had a higher risk of recent TB and therefore, could be an entry point for targeted interventions.

5.2. Profile and frequency of mutations conferring DR

For anti-TB drugs to be effective, early diagnosis and having effective drugs against the infecting MTB isolate is essential to improve the cure rate of the patients and hinder further transmission of the TB disease. Furthermore, identifying the mutations associated with anti-TB drug resistance is essential to properly manage DR-TB patients. LPAs come at the forefront in undertaking these tasks, especially in developing countries such as Ethiopia, for some key anti-TB drugs used in the treatment of drug susceptible and DR-TB.

Mutations in the 81-bp region (codons 507-533) of the *rpoB* gene harbor over 95% of RIF resistance in MTB isolates and high-level RIF resistance is usually associated with point mutations in 531, 526 and 516 codons (Hirani et al., 2020b). In the current study, among 88 RIF resistant MTB isolates, the most common gene mutation (59.1%) associated with RIF resistance was at codon S531L. This mutation was reported as a predominant mutation of the *rpoB* gene causing RIF resistance by various studies previously conducted in Ethiopia which include Jigjiga town (Brhane et al., 2017), Amhara region (Tessema et al., 2012), Ethiopia (Reta et al., 2021), St. Peter's hospital, Addis Ababa, Ethiopia (Damena et al., 2019), Southwest Ethiopia (Tadesse et al., 2017), and Tigray region (Welekidan et al., 2021). In agreement with our finding, higher frequency of mutation at codon S531L was reported in other countries such as Sudan (64.1%), India (62.3%), Iran (66%), Pakistan (64%), and China (58.2%) (Elbir & Ibrahim, 2014; Farooqi et al., 2012; Hamed et al., 2021; Jian et al., 2018; Maurya et al., 2013).

Although in less frequency, the second most common mutation D516V (14.8%) in this study was previously reported in Ethiopia (Damena et al., 2019; Reta et al., 2021), India (Alvarez-Uria & Reddy, 2018) and Sudan (Elbir & Ibrahim, 2014). However, higher or similar proportion to our findings was reported in Angola,17.2% (Rando-Segura et al., 2021), India,17.7% (Maurya et al., 2013), China,10.1% (Jian et al., 2018) and Ecuador, 28.6% (Franco-Sotomayor et al., 2019). Eleven isolates (12.5%) showed mutations at codon 526 in which 9.1% were at H526Y and 3.4% at H526D. Comparable findings were reported in Ethiopia (Abate et al., 2014) and India (Maurya et al., 2013).

In the current study, 13.6% of isolates were classified as RIF resistant based on only the lack of WT probe hybridization. This proportion of isolates with unknown mutations is similar to other studies from St. Peter's TB specialized hospital, Addis Ababa, Ethiopia,15.8% (Abate et al., 2014) and Southwest Ethiopia, 14.7% (Tadesse et al., 2016) and a multicenter study in India, South Africa and Moldova, 13% (Seifert et al., 2016).

INH resistance is mainly caused by mutations in the *katG* and *inhA* genes; with 50-95% of INH-resistant isolates having *kat*G S315 mutations, depending on geographical distribution (Bostanabad et al., 2008; Rueda et al., 2015; Zhang & Yew, 2009). Similarly, most of the INH resistant isolates (96.5%) in our study had mutations in the *katG* gene (S315T1/T2) while the remaining 3.5% had mutations in *inh*A (c15T) promoter region. In agreement with our findings, a meta-analyses study that examined INH conferring mutations (Reta et al., 2021) reported a prevalence of 95.8% for *katG*315 mutation and 5.9% for *inh*A promoter region mutation. High-level INH resistance causing mutation (S315T1) (van Soolingen et al., 2000) was the most frequent (91.8%) in our study and other studies conducted in Ethiopia (Biadglegne et al., 2013; Brhane et al., 2017; Damena

et al., 2019; Tadesse et al., 2016; Tessema et al., 2012). Furthermore, the *kat*G 315 mutations reported to be frequent in MDR-TB patients (Tilahun et al., 2020; van Soolingen et al., 2000) were exclusively found in MDR-TB isolates in the current study.

Mutations in the *inh*A promoter region which are associated with low-level INH resistance are usually less frequent when compared with *kat*G mutations (Zhang & Yew, 2009). In this study, we found only three INH resistant isolates (all monoresistant) with mutations at codon C15T of the *inh*A promoter gene. In earlier studies from Ethiopia, mutations in *inh*A promoter region were mostly in INH monoresistant isolates (Damena et al., 2019; Tadesse et al., 2016; Tilahun et al., 2020). Other studies also reported no or low proportion of mutation in the *inh*A promotor region (Abate et al., 2014; Alelign et al., 2019; Biadglegne et al., 2013; Damena et al., 2019; Tessema et al., 2012).

In Ethiopia, the indiscriminate use of FLQs for various indications might have led to the development of drug resistance against these key drugs (Agonafir et al., 2010; Diriba et al., 2022; Shibabaw et al., 2020). Mutations in the *gyrA* and *gyrB* gyrase genes especially at codons 90, 91 and 94 of *gyrA* (termed quinoline resistance-determining region, QRDR) are responsible for FQLs resistance in MTB isolates (Avalos et al., 2015). In this study, *gyrA* mutation at codon A90V was the most common (42.9%) among the FLQs resistant isolates, which is in agreement with a laboratory-based surveillance study (Diriba et al., 2022) in Ethiopia and a report from Morocco (Chaoui et al., 2018). Supporting our findings, D94N/D94Y was recently reported to be the second most common *gyrA* mutation in Ethiopia (Diriba et al., 2022; Welekidan et al., 2021). Various investigations have indicated that D94G mutation is predominant across the corners of

the globe (Ajbani et al., 2012; Avalos et al., 2015; Jnawali et al., 2013; Jou et al., 2019; Kabir et al., 2020; Singh et al., 2021). In our study, no mutation related to *gyrB* was observed, which is in concordance with previous studies in Ethiopia (Diriba et al., 2022; Welekidan et al., 2021).

Of note, one isolate had a WT probe hybridization and A90V and D94N/Y mutations indicating heteroresistance (coexistence of susceptible and resistant strain in a single specimen) (Singhal et al., 2016). Superinfection or reinfection by a second strain, mixed infection or within host evolution of strains could result in heteroresistance (Ford et al., 2012). As an intermediate stage of full resistance, the detection of heteroresistant mutations is important in guiding the provision of proper treatment regimen (Abakur et al., 2020; Liang et al., 2018).

Resistance to SLIDs is mostly associated with *rrs* A1401G mutation (Georghiou et al., 2012). Similarly, the A1401G mutation was reported as the most frequent in Ethiopia (Diriba et al., 2022). However, in the current study, three isolates had unknown *rrs* mutation that results in cross-resistance to kanamycin, capreomycin and viomycin (HAIN Lifescience, 2015b). It is well known that kanamycin and capreomycin are no more used in the treatment of DR-TB (WHO, 2021).

5.3. Molecular characterization of MDR-MTB isolates

In this study, 79 MDR MTBC isolates collected from central, eastern and southeastern Ethiopia were genotyped by spoligotyping to describe the population structure of circulating strains. Additionally, 22 selected isolates (out of the 79) from East Ethiopia were characterized using WGS to investigate strain types, transmission dynamics, and genomic drug resistance signatures to anti-TB drugs.

Spoligotyping analysis of the 79 MDR MTB isolates unveiled that a high proportion of the study isolates (74.7%) were clustered. Similar clustering rate (76%) was documented in an earlier study at St. Peter's TB specialized hospital in Addis Ababa (Diriba et al., 2013). While acknowledging the limitations of spoligotyping in distinguishing closely related MTBC strains (Driscoll, 2009), the high clustering rate underscores potential ongoing transmission of MDR-TB in the study area. Furthermore, our findings showed multiple lineages (L1-L7), with the majority falling under EA/L4 (70.9%) and the second-largest group belonging to EAI/L3 (20.3%), indicating the importance of EA/L4 and EAI/L3 in MDR-TB transmission in the study area. The third most prevalent lineage was EAS/L2, which was detected in isolates from East Ethiopia (n=4, 5.1%). The remaining included ETH/L7, IO/L1, and MA, with proportions of 1.3% each. To our knowledge, this is the first study that reported EAS/L2 and ETH/L7 in MDR MTBC isolates. On the contrary, IO/L1 (Diriba et al., 2013) and MA (Mollalign et al., 2023) were previously reported.

Unlike the outcomes of our investigation, a prior study conducted at St. Peter's TB specialized hospital on MDR MTBC isolates reported a proportionate distribution

between EA/L4 and IO/L1, followed by EAI/L3 (Diriba et al., 2013). Two studies from the Amhara region also reported the predominance of EA/L4, followed by EAI/L3, in MDR MTBC isolates, which is consistent with our findings (Mekonnen et al., 2023; Shibabaw et al., 2023).

Both EA/L4 and EAI/L3 are commonly identified MTBC lineages in Ethiopia. However, EA/L4 is particularly prevalent in Ethiopia and globally, having a significant impact on millions of lives (Brynildsrud et al., 2018; O'Neill et al., 2019; Tulu & Ameni, 2018). Studies have shown that the increase in MDR-TB caused by EA/L4 strains in recent years has primarily been a local phenomenon, with resistant clones emerging repeatedly in various locations and limited evidence of transnational transmission occurring (Brynildsrud et al., 2018; Ritacco et al., 2012). In line with this evidence, clusters of EA/L4 sub-lineages such as SIT49/T3_ETH, SIT53/T1 and Haarlem were previously found in MDR MTBC isolates from different parts of Ethiopia (Agonafir et al., 2010; Bekele et al., 2018; Diriba et al., 2013; Mekonnen et al., 2023; Tessema et al., 2013). Similarly, distinct clusters of strains SIT149/T3_ETH, SIT53/T1 and SIT41/Turkey of EA/L4 were detected in the current study. Notably, the predominant and locally adapted SIT49/T3_ETH, assumed to have likely arrived during a large-scale human migration around 3000 years ago (Comas et al., 2015), was the most frequent among all MDR MTBC isolates in our study. This strain represents a hallmark of TB epidemiology in Ethiopia, reflecting the historical context of TB dissemination and the persistence of specific lineages in the region.

EAI/L3 strains of the MTBC, which were introduced to Ethiopia, Kenya and Sudan, through ancient exchanges and migrations between South Asia and Eastern Africa have

been found to exhibit multidrug resistance in varying proportions (Shuaib et al., 2022). A previous study conducted in Ethiopia (Diriba et al., 2013) found that the SIT21/CAS1_kili strain was the most prevalent among MDR MTBC isolates. Two studies from Zambia and Djibouti also reported the predominance of SIT21/CAS1_kili (Millán-Lou et al., 2016; Solo et al., 2021) in MDR MTBC isolates. In concordance with the present study, a prior study in Ethiopia (Agonafir et al., 2010) also revealed that CAS1_kili was the second most common strain among MDR MTBC isolates.

Affirming the spoligotyping-based findings, further analysis of 22 MDR MTBC isolates using WGS from East Ethiopia also showed that EA/L4 (n=11, 50.0%) and EAI/L3 (n=7, 31.8%) were the most common genotypes, followed by EAS/L2 (n=4, 18.2%). Of these, over 90% of the EA/L4 strains (90.9%) and all of the EAI/L3 and EAS/L2 strains belonged to the L4.2.2, L3.3.1, and L2.2.1 sub-lineages, respectively. In a recent WGS-based MDR-TB study conducted in the Amhara region, a comparable proportion (92.0%) of L4.2.2 and a lower proportion of L3.1.1 (47%) were reported (Shibabaw et al., 2023). In contrast to our findings, the Amhara region study and other similar studies (Agonafir et al., 2010; Diriba et al., 2013; Mekonnen et al., 2023) did not report the presence of the EAS/L2 Beijing strains among MDR MTBC isolates in Ethiopia.

Remarkably, the cgMLST cluster analysis yielded three recent transmission clusters (with a maximum threshold of 5 alleles), each consisting of two isolates, belonging to L2.2.1 (cluster 1), L3.3.1 (cluster 3), and L4.2.2 (cluster 2), despite the limited sample size. Notably, five of these strains originated from Diredawa, while the other was from Garamuleta, a town located 99.4 km away. Within the Beijing L2.2.1 clone, both BF26 and BF60 exhibited shared mutations at *rpoB*_D435V and *katG*_S315T. Similarly, in

clusters 2 and 3, shared mutations were observed, albeit with slight differences in drug resistance polymorphisms. Specifically, BF1 in cluster 2 harbored a compensatory *rpoC*_G332R mutation absent in BF16, whereas B16 displayed additional *embA*_c.-12C>T and *tlyA*_c.358_359insG mutations. Within cluster 3, BF58 demonstrated an additional *gyrA*_D94N mutation compared to BF12. These findings underscore an ongoing transmission of MDR-TB via multiple clones in Diredawa and its surrounding areas, which necessitates further investigation through a population-based molecular epidemiological study to confirm these findings.

In Ethiopia, multiple studies have elucidated polymorphisms in genes related to drug resistance to certain anti-TB drugs through the use of commercial tools. Recent studies, leveraging WGS, has expanded this understanding (Mekonnen et al., 2023; Welekidan et al., 2021). By enabling the detection of drug resistance across a broader spectrum of anti-TB drugs, WGS can provide insights into the variations in mutations associated with drug resistance across diverse geographic regions of the country.

In agreement with a previous review (Reta et al., 2021), *rpoB*_S450L and *katG*_S315T mutations, which are characterized by low or no cost of fitness (Gagneux et al., 2006; Pym et al., 2002), were predominant among MDR MTBC isolates in East Ethiopia. Interestingly, one isolate harbored *rpoB*_S450L and *rpoC*_G332R compensatory mutation. Several studies have established a significant connection between *rpoB*_S450L and *rpoC* mutations, suggesting that *rpoC* mutations may help counteract the fitness challenges posed by RIF resistance in MTB, potentially facilitating its spread (Claassens et al., 2022; Wang et al., 2020). Additionally, these compensatory mutations play a crucial role on a global scale, allowing the formation and persistence of extensive

transmission networks among RIF-resistant, MDR and pre-XDR strains of MTB (Emane et al., 2021; Merker et al., 2018). We also detected disputed *rpoB* mutations such as H450C, H445C, H445G which have been reported to confer highly discordant RIF results by phenotypic drug susceptibility testing and may have a negative impact on patient management (Al-Mutairi et al., 2019; Berrada et al., 2016; P. Hu et al., 2019; Miotto et al., 2018).

Mutations in the *embCAB* operon are thought to be the underlying cause of approximately 70% of EMB drug resistant cases (Xiang et al., 2021). In the present study, mutations in the *embB* and *embA* genes were identified. A significant proportion of these (50%) were nucleotide substitutions at codon 306 of the embB gene (*embB_306)*, including *embB*_M306V (35.7%) and *embB*_M306I (14.3%). The *embB_306* codon mutations are the most common among EMB-resistant isolates (Mohammadi et al., 2020; Sun et al., 2017; Yaşar et al., 2023); and may serve as a potential marker for EMB resistance and predictor of extensive MTB drug resistance (Hazbón et al., 2005; Li et al., 2016; Munir et al., 2017). Mutations at codons *embB*_M306V, *embB*_M306I, *embA*_c.-12C>T and the double mutation (*embA*_c.-16C>T with *embB*_M306I) detected in our study were reported to lead to an increase in EMB MIC levels (Sun et al., 2017; Xiao et al., 2023).

Despite its limited use in TB treatment, 50% (n=11) of the MDR MTBC isolates subjected to WGS were resistant to STM. Majority of them (54.6%) had mutations in *gid* (102_103insG) and a *combination* of mutations in *gid and rrs* (c.386_386del and A514C) genes. Interestingly, unlike the previous reports (Osei Sekyere et al., 2019; Rocha et al.,

2021; Shrestha et al., 2020; Wang et al., 2022; Welekidan et al., 2021), the widely reported *rpsL* (K43R and K88R) mutations were detected in one isolate each.

The mutations *pncA*_T135I (28.6%) and *pncA*_L27P (28.6%), which were previously reported to cause PZA resistance (CRyPTIC Consortium and the 100,000 Genomes Project et al., 2018; Yadon et al., 2017), were the most common mutations in the current study. Other mutations such as *pncA*_T135P, *pncA*_V139G, and *pncA*_192_193insA were also identified. Most ETO-resistant isolates show mutations in *ethA, mshA, ndh and inhA* genes. However, the connection between genotype and ETO-resistant phenotype is still being investigated (Rueda et al., 2015; Ushtanit et al., 2022). All three ETO-resistant isolates in the current study showed *ethA* mutations. For FLQs-resistant strains, previously known mutations at codon 94 of the *gyrA* gene (D94G and D94N) and double point mutations at codons *gyrA*_A90V and *gyrA*_D94A were detected. D94G and D94N mutations are common (Li et al., 2014) while the double codons mutation (A90V and D94A) is rare (Andreevskaya et al., 2022; Yin & Yu, 2010). Research suggests that *gyrA* mutations and FLQ resistance exhibit regional diversity which may be attributable to a range of social and geographic factors shaping transmission environments and selective pressures. Alternatively, the use of distinct FLQ-containing treatment regimens in various regions may contribute to different drug-based selection pressures (Avalos et al., 2015).

5.4. Limitation of the Study

Our study is not without limitations. The convenient selection of isolates from specific diagnostic centers may pose selection bias, and the relatively small number of isolates could affect the representativeness of the study results. Additionally, our focus on central, eastern, and southeastern Ethiopia may limit the applicability of the findings to other regions of the country, and the concentration of WGS analysis on selected isolates from East Ethiopia might introduce bias and reduce the representativeness of the outcomes. Furthermore, the absence of epidemiological links as well as other clinical information present challenges in establishing transmission patterns, and the smaller sample size of isolates undergoing WGS analysis may restrict the scope of genetic characterization, necessitating prudence when generalizing the results to the broader population of MDR-TB strains in Ethiopia.

CHAPTER 6

Conclusion and Recommendations

6. Conclusion and recommendations

6.1. Genetic diversity of MTB isolates from the central, eastern and southeastern Ethiopia

A total of 223 MTBC culture isolates were characterized using spoligotyping to describe the genetic diversity of MTBC in Central, Eastern, and Southeastern Ethiopia. The study identified six major lineages: EA/L4, EAI/L3, EA/L2, IO/L1, MA, and ETH/L7. The majority (94.6 %) of the isolates were EA/L4 (75.3 %) and EAI/L3 (19.3 %). The study revealed 77 different spoligotype patterns, with 27 being unique and 15 clustered distributed across different geographic locations. Among the clustered isolates (n=159), spoligotype SIT149/T3_ETH (n=49) was the most predominant among the clustered isolates followed by SIT53/T1 (n = 33), SIT21/CAS1_Kili (n = 24) and SIT41/Turkey (n=11). Additionally, a rare Beijing spoligotype pattern, SIT541, was identified in Eastern Ethiopia. Age group (25–34 years) was significantly associated with clustering. The findings indicate significant genetic diversity of MTBC in the study area, despite the dominance of T (Euro-American/Lineage 4) and CAS (East-African Indian/Lineage 3) families. The predominance of EA/L4, comprising three-fourths of the isolates, highlights its impact on the TB landscape of Ethiopia. Moreover, the identification of the Beijing strain, particularly the rare SIT541 spoligotype cluster in eastern Ethiopia, calls for enhanced surveillance.

The high genetic diversity and presence of multiple lineages suggest that TB control efforts need to be tailored to address the specific characteristics and transmission dynamics of these strains. The dominance of the EA/L4 and the detection of the rare

Beijing strain indicate potential areas for targeted intervention as well as continuous monitoring to adapt to the evolving epidemiological landscape of TB in Ethiopia. Future studies should focus on large-scale, longitudinal surveillance using advanced molecular tools such as WGS to capture comprehensive data on MTBC genetic diversity and transmission dynamics across Ethiopia. Investigating the socio-economic and environmental factors contributing to the predominance of specific lineages can further inform tailored intervention strategies. Collaborative efforts between local and international research institutions can enhance the capacity for robust TB research and control in Ethiopia.

6.2. Profile and frequency of mutations conferring DR-TB in the central, southeastern and eastern Ethiopia

The current study aimed to determine the frequency and type of mutations associated with resistance to key anti-TB drugs: RIF, INH, FLQs and SLIDs. The study found that 39.3% of the isolates exhibited mutations conferring resistance to RIF, 38.0% to INH, 9.1% to FLQs, and 3.9% to SLIDs. Canonical drug resistance-conferring mutations in the *rpoB* (S531L, 59.1%), *katG* (S315T, 96.5%), and *gyrA* (A90V, 42.1%) genes were the most frequent in isolates resistant to RIF, INH, and FLQs, respectively. Notably, more than 10% of RIF-resistant isolates presented unknown *rpoB* mutations that could complicate the decision-making process in patient management. Similarly, all SLID-resistant isolates had unknown mutations in the *rrs* gene.

The findings of this study have significant implications for TB prevention and care, emphasizing the need for enhanced diagnostic accuracy, personalized treatment

regimens, regional adaptation of diagnostic tools, and informed public health strategies. The high proportion of canonical mutations such as *rpoB* S531L, *katG* S315T, and *gyrA* A90V signifies the reliability of these markers for detecting drug resistance using molecular diagnostic tools. However, the presence of unknown mutations, especially in RIF and SLID-resistant isolates, highlights the limitations of current diagnostic assays and the need for continual updates to include novel resistance-conferring mutations. Identifying specific mutations associated with drug resistance can guide the selection of effective treatment regimens, for example, using high-dose INH or alternative drugs that bypass resistance mechanisms identified in the inhA promoter region of INH monoresistant isolates. Geographic variability in mutation types and frequencies necessitates the adaptation of diagnostic tools to regional genetic profiles, enhancing the sensitivity and specificity of molecular diagnostics in detecting drug-resistant TB strains prevalent in specific areas. Understanding the mutation landscape aids in monitoring the spread of drug-resistant TB, evaluating the effectiveness of public health interventions, and identifying high-risk populations and transmission hotspots, thereby facilitating targeted control measures.

6.3. Molecular characterization of MDR MTBC isolates in central, eastern and southeastern Ethiopia

The continued emergence and spread of drug-resistant TB strains, particularly MDR-TB, represents a formidable challenge to global TB control efforts. Despite some progress in reducing MDR-TB rates, Ethiopia still grapples with substantial hurdles related to MDR-TB. Genetic characterization of MDR MTBC isolates is fundamental for the effective

management of drug-resistant TB. It aids in the rapid identification of resistant strains, informs treatment decisions, and assists in monitoring the spread of resistance. This study aimed to describe the genetic diversity, transmission dynamics, and drug resistance characteristics of MDR-MTBC isolates in central, eastern and southeastern Ethiopia. The current study included 79 MDR-TB isolates. Spoligotyping revealed 25 distinct spoligotype patterns. Sixteen patterns, containing 70 isolates (88.6%), matched those in the international database, while nine patterns (11.4%) were new. The most common lineage was EA/L4 (70.9%), followed by EAI/L3 (20.3%), with smaller proportions of EAS/L2, ETH/L7, IO/L1, and MA lineages. Sub-lineage analysis highlighted SIT149/T3-ETH (32.9%) and SIT21/CAS1_Kili (17.7%) as predominant. WGS analysis of isolates from East Ethiopia also revealed the predominance of EA/L4 and EAI/L3. It identified prevalent mutations in *rpoB, katG, embB, pncA, gyrA, ethA*, and other genes, which contribute to drug resistance. Moreover, WGS revealed recent transmission clusters, indicating ongoing local transmission. The study underscores the critical need for ongoing genetic surveillance of MDR-TB in Ethiopia, revealing various lineages and spoligotype patterns, which highlight the genetic diversity and complexity of MDR-TB in the region. The predominance of the EA/L4 and EAI/L3 lineages, along with the detection of lineages like EAS/L2 and ETH/L7, which have never been previously reported in Ethiopian MDR MTBC isolates, expands our comprehension of the diversity of MDR-TB strains in Ethiopia. Moreover, findings from WGS, which identified significant mutations in genes such as *rpoB, katG, embB, pncA, gyrA, ethA* and other genes, highlight the importance of comprehensive genomic approaches over conventional methods. These genetic insights can inform the refinement of rapid molecular diagnostic

tests to include a broader range of mutations, improving the accuracy of resistance detection and guiding effective treatment plans. Additionally, the high proportion of clustered isolates and identification of recent transmission clusters emphasizes the ongoing transmission of MDR-TB, necessitating strengthened public health interventions to curb further spread. Enhanced infection control measures, community-based surveillance, and targeted treatment programs are essential to manage and prevent MDR-TB transmission. Future investigations should incorporate WGS analysis, carry out longitudinal studies, perform thorough epidemiological analyses, assess intervention strategies, and promote collaboration and capacity building among local and international research bodies. By tackling these research voids and executing tailored interventions informed by genetic understandings, significant strides can be made in managing and curtailing MDR-TB in Ethiopia and globally.

To conclude, the findings derived from the aforementioned studies are poised to make a significant impact on the trajectory of TB/MDR-TB control initiatives in Ethiopia. Priority should be given to strengthening the capacity of laboratories in Ethiopia to conduct molecular testing, including WGS, and to enhance surveillance for TB and DR-TB.

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ORIGINAL RESEARCH

Profile and Frequency of Mutations Conferring Drug-Resistant Tuberculosis in the Central, Southeastern and Eastern Ethiopia

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Purpose: Advances in molecular tools that assess genes harboring drug resistance mutations have greatly improved the detection and treatment of drug-resistant tuberculosis (DR-TB). This study was conducted to determine the frequency and type of mutations that are responsible for resistance to rifampicin (RIF), isoniazid (INH), fluoroquinolones (FLQs) and second-line injectable drugs (SLIDs) in *Mycobacterium tuberculosis* (MTB) isolates obtained from culture-positive pulmonary tuberculosis (TB) patients in the central, southeastern and eastern Ethiopia.

Patients and Methods: In total, 224 stored culture-positive MTB isolates from pulmonary TB patients referred to Adama and Harar regional TB laboratories between August 2018 and January 2019 were assessed for mutations conferring RIF, INH, FLQs and SLIDs resistance using GenoType®MTBDRplus (MTBDRplus) and GenoType®MTBDRsl (MTBDRsl).

Results: RIF, INH, FLQs and SLIDs resistance-conferring mutations were identified in 88/224 (39.3%), 85/224 (38.0%), 7/77 (9.1%), and 3/77% (3.9%) of MTB isolates, respectively. Mutation codons *rpoB* S531L (59.1%) for RIF, *katG* S315T (96.5%) for INH, *gyrA* A90V (42.1%) for FLQs and WT1 *rrs* (100%) for SLIDs were observed in the majority of the isolates tested. Over a 10th of *rpoB* mutations detected in the current study were unknown.

Conclusion: In this study, the most common mutations conferring drug resistance to RIF, INH, FLQs were identified. However, a significant proportion of RIF-resistant isolates manifested unknown *rpoB* mutations. Similarly, although few in number, all SLID-resistant isolates had unknown *rrs* mutations. To further elucidate the entire spectrum of mutations, tool such as whole-genome sequencing is imperative. Furthermore, the expansion of molecular drug susceptibility testing services is critical for tailoring patient treatment and preventing disease transmission.

Keywords: drug resistance, Ethiopia, *line probe assay*, mutation, tuberculosis

Introduction

Though curable and preventable, with an estimated 9.9 million new cases and 1.3 million deaths in 2020, TB is still one of the main causes of death globally. DR-TB especially resistant to INH and RIF, termed as multidrug-resistant TB (MDR-TB), poses a great threat to the public. Worldwide, 132,222 individuals were reported to have MDR-TB and rifampicin-resistant tuberculosis (RR-TB) in 2020.[1] Although Ethiopia achieved the End TB Strategy milestone of a 20% reduction in the TB incidence rate from 2015 to 2020, it is still 1 of the 30 high burden countries heavily affected by TB and TB/HIV.[1] Furthermore, despite the fact that Ethiopia is one of the countries that has transitioned out of the 30 high MDR/RR-TB countries,[1] DR-TB is still a problem where 2.8% of the new and 18.6% of previously treated cases were reported to have MDR-TB.[2]

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Genetic diversity of *Mycobacterium tuberculosis* isolates from the central, eastern and southeastern Ethiopia

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ABSTRACT

Introduction: The population structure of *Mycobacterium tuberculosis* complex (MTBC) in Ethiopia is diverse but dominated by Euro-American (Lineage 4) and East-African-Indian (Lineage 3) lineages. The objective of this study was to describe the genetic diversity of MTBC isolates in Central, Eastern and Southeastern Ethiopia.

Methods: A total of 223 MTBC culture isolates obtained from patients referred to Adama and Harar TB reference laboratories were spoligotyped. Demographic and clinical characteristics were collected.

Results: Six major lineages: Euro-American (Lineage 4), East-African-Indian (Lineage 3), East Asian (Lineage 2), Indo-Oceanic (Lineage 1), *Mycobacterium africanum* (Lineage 5 and Lineage 6) and Ethiopian (Lineage 7) were identified. The majority (94.6 %) of the isolates were Euro-American and East-African-Indian, with proportions of 75.3 % and 19.3 %, respectively. Overall, 77 different spoligotype patterns were identified of which 42 were registered in the SITVIT2 database. Of these, 27 spoligotypes were unique, while 15 were clustered with 2–49 isolates. SIT149/T3_ETH (n = 49), SIT53/T1 (n = 33), SIT21/CAS1_Kili (n = 24) and SIT41/Turkey (n = 11) were the dominant spoligotypes. A rare Beijing spoligotype pattern, SIT541, has also been identified in Eastern Ethiopia. The overall clustering rate of sub-lineages with known SIT was 71.3 %. Age group (25–34) was significantly associated with clustering.

Conclusion: We found a heterogeneous population structure of MTBC dominated by T and CAS families, and the Euro-American lineage. The identification of the Beijing strain, particularly the rare SIT541 spoligotype in Eastern Ethiopia, warrants a heightened surveillance plan, as little is known about this genotype. A large-scale investigation utilizing a tool with superior discriminatory power, such as whole genome sequencing, is necessary to gain a thorough understanding of the genetic diversity of MTBC in the nation, which would help direct the overall control efforts.

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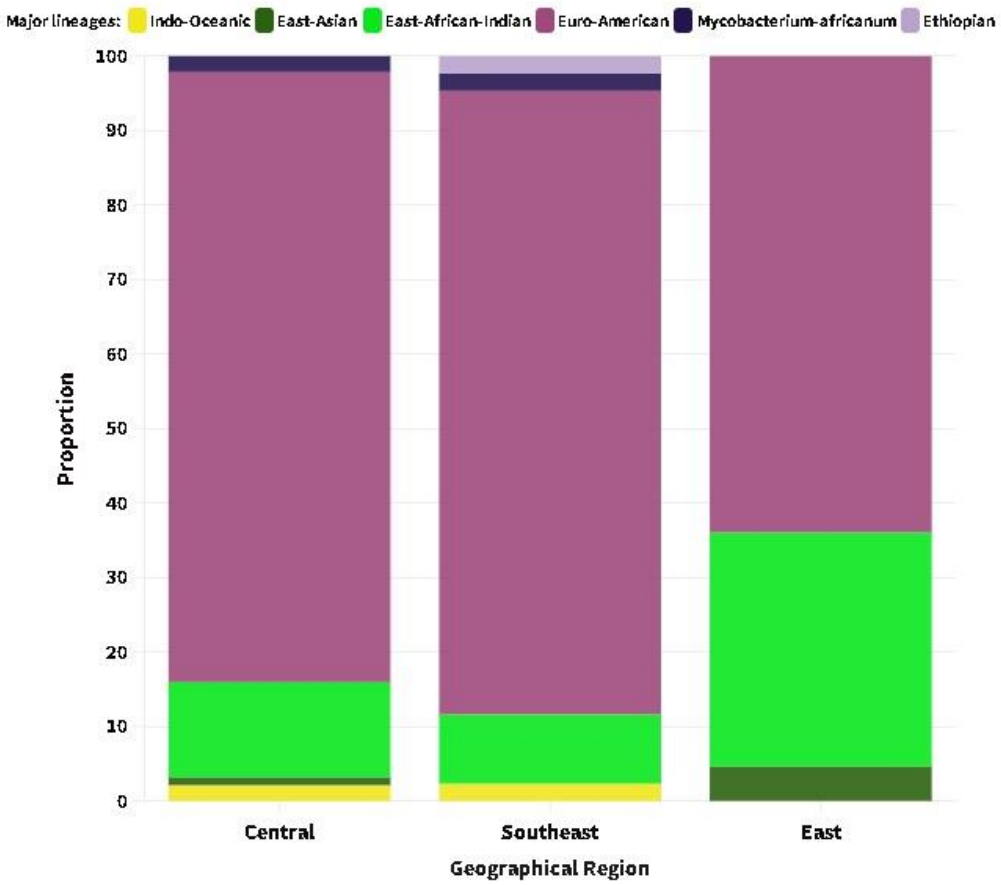
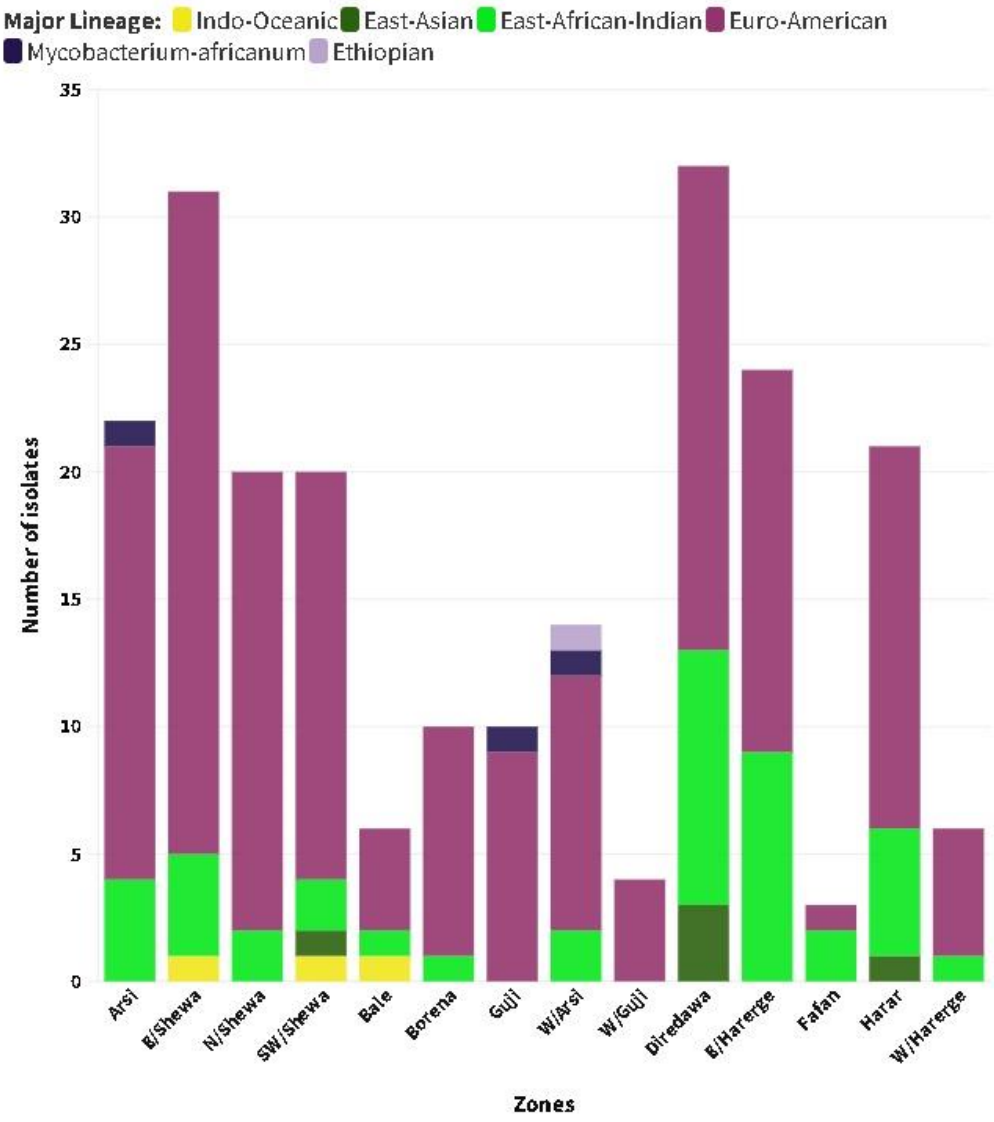
LIST OF APPENDICES

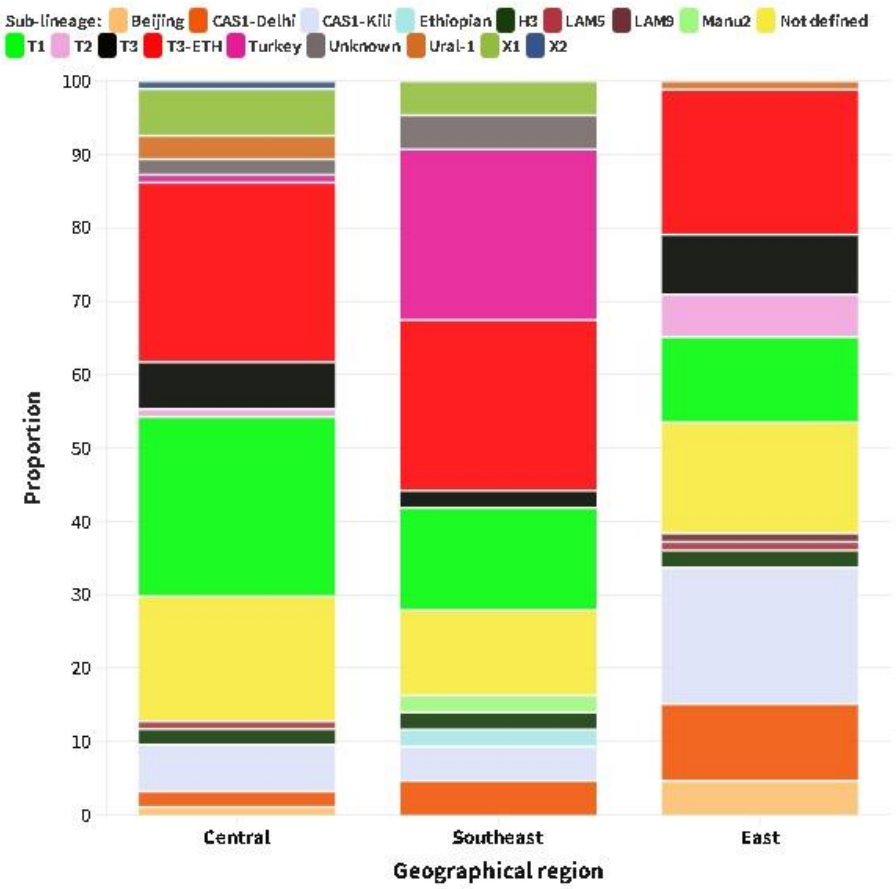
Appendix 1: Distribution of MTBC major lineages (by geographic region) in the central, eastern and southeastern Ethiopia



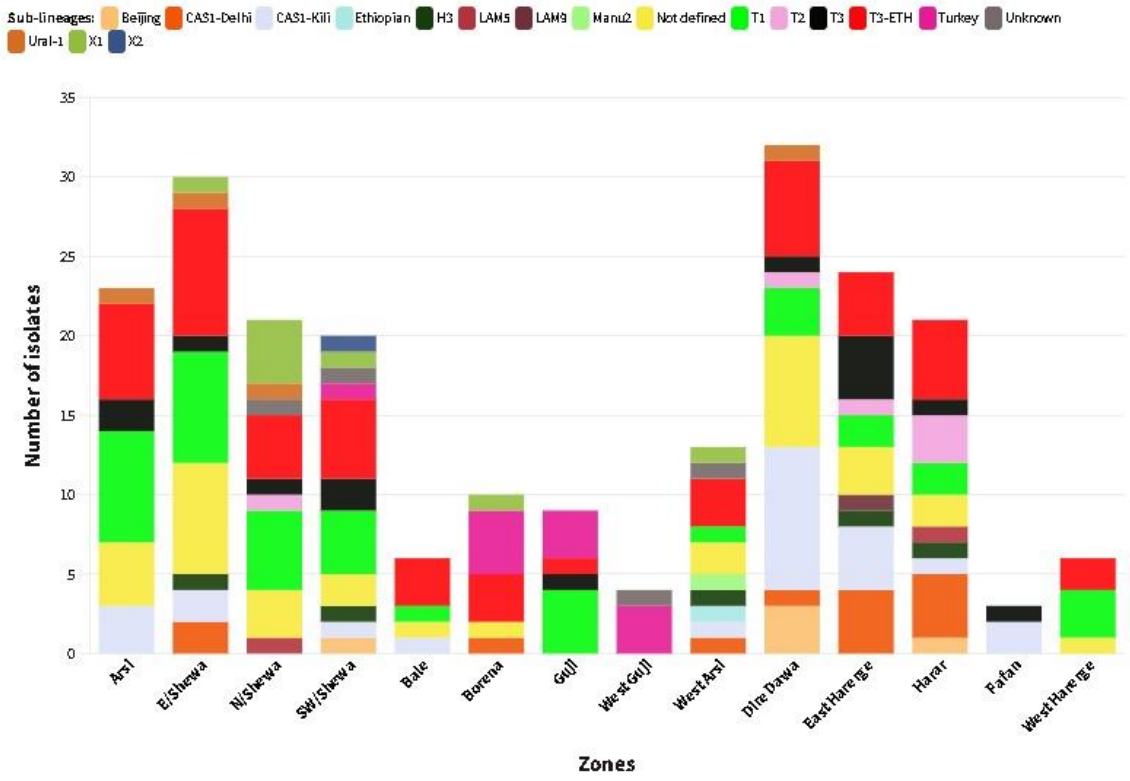
Appendix 2: Distribution of MTBC major lineages (by zones) in the central, eastern and southeastern Ethiopia.



Appendix 3: Distribution of MTBC sub-lineages (by geographical region) in the central, eastern and southeastern Ethiopia



Appendix 4: Distribution of MTBC sub-lineages (by zones) in eastern, southeastern and eastern Ethiopia.



Appendix 5: Description of WGS based lineages and drug resistance prediction of 22 MDR M. tuberculosis isolates in East Ethiopia.

Isolate	Sub-lineage	Spoligotype	Genotypic drug resistance pattern	Mutation
BF1	L4.2.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB*_S450L, *rpoC*_G332R+ *katG*-S315T+ *gid*_c.102_103insG
BF-2	L3.1.1	CAS1-kili	RIF+INH+EMB+STM	*rpoB*_H445Y, *rpoB*_T427P + *katG*_S315T + *embB*_M306V+ *rpsL*_K43R
BF-3	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM	*rpoB*_S450L + *katG*_S315T+ *pncA*_T135P + *embB*_M306I, *embB*_G406A+ *gid*_c.386_386del
BF-4	L4.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB*_S450L + *katG*_S315T+ *gid*_c.102_103insG
BF-8	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM+ ETH	rpoB_S450L + katG_S315T+ embB_M306V + pncA_c.192_193insA + *rpsL*_K88R + ethA_Chromosome:g.4326474_4326616del/ethA c.858_999del
BF-12	L3.1.1	CAS1_kili	RIF+INH+PZA+EMB+STM+ ETO	*rpoB*_H445G + *katG*_S315T + *pncA*_L27P+ *embB*_M306I, *embB*_G406S+ *gid*_c.386delG+ *rrs*_A514C + *ethA*_c.-11A>G
BF-15	L3.1.1	CAS1_kili	RIF+INH+EMB	*rpoB*_S450L+*katG*_S315T+ *embB*_M306I
BF-16	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+STM	*rpoB*_S450L+ *katG*_S315T+ *embA*_c.-12C>T+ *gid*_c.102_103insG
BF-20	L4.8	T1; T2; T3: T5	RIF+INH+EMB	*rpoB*_S450S/C*+*katG*_S315T+ *embB*_M306V
BF-21	L3.1.1	CAS1-kili	RIF+INH+EMB+STM	*rpoB*_H445D +*katG*_S315T+ *embB*_M306I+ *gid*_c.386_386del, *rrs*_A514C
BF-24	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+FLQ+ETO**	*rpoB*_S450L+*katG*_S315T+*embB*_M306V+*gyrA*_D94A,*gyrA*_A90V+*ethA*_M1R
BF-26	L2.2.1	Beijing	RIF+INH	*rpoB*_D435V +*katG*_S315T
BF37	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+FLQ+ETO**	*rpoB*_H445C + *katG*_S315T + *embA_c.-16C>T*, *embB*_M306I+ *gyrA*_D94G+ *ethA*_M1R
BF-38	L4.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB*_S450L +*katG*S315T + *rrs*_A514C
BF-39	L2.2.1	Beijing	RIF+INH	*rpoB*_D435V + *katG*_S315T
BF-40	L4.2.2	T;LAM7-TUR	RIF+INH	*rpoB*_S450L + *katG*_S315T
BF-42	L4.2.2	T;LAM7-TUR	RIF+INH+PZA+EMB	*rpoB*_S450L +*katG*_S315T+ *pncA*_T135I+ *embB*_H1002R
BF-43	L2.2.1	Beijing	RIF+INH+PZA+ EMB+ STM	*rpoB*_D435V +*katG*_S315T+ *pncA*_V139G+ *embB*_M306V + *rrs*_C517T

BF-44	L4.2.2	T;LAM7-TUR	RIF+INH	*rpoB*_H445C + *katG*_S315T
BF-45	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+ PZA	*rpoB*_S450L + *katG*_S315T+ *embB*_H1002R + *pncA*_T135I
BF-58	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM+FLQ+ETO+CAP**	*rpoB*_H445G+*katG*_S315T+*pncA*_L27P+*embB*_M306I, *embB*_G406S+*gid*_c.386_386del+rrs_A514C+*gyrA*_D94N+ *ethA*_c.11A>G+ *tlyA*_c.358_359insG
BF-60	L2.2.1	Beijing	RIF+INH	*rpoB*_D435V + *katG*_S315T
BF1	L4.2.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB_S450L, rpoC_G332R+ katG-S315T+ gid_c.102_103insG*
BF-2	L3.1.1	CAS1-kili	RIF+INH+EMB+STM	*rpoB_H445Y, rpoB_T427P + katG_S315T + embB_M306V+ rpsL_K43R*
BF-3	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM	*rpoB_S450L + katG_S315T+ pncA_T135P + embB_M306I, embB_G406A+ gid_c.386_386del*
BF-4	L4.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB_S450L + katG_S315T+ gid_c.102_103insG*
BF-8	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM+ETH	*rpoB_S450L + katG_S315T+ embB_M306V + pncA_c.192_193insA + rpsL_K88R + ethA_Chromosome:g.4326474_4326616del/ethA c.858_999del*
BF-12	L3.1.1	CAS1_kili	RIF+INH+PZA+EMB+STM+ETO	*rpoB_H445G + katG_S315T + pncA_L27P+ embB_M306I, embB_G406S+ gid_c.386delG+ rrs_A514C + ethA_c.-11A>G*
BF-15	L3.1.1	CAS1_kili	RIF+INH+EMB	*rpoB_S450L+katG_S315T+ embB_M306I*
BF-16	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+STM	*rpoB_S450L+ katG_S315T+ embA_c.-12C>T+ gid_c.102_103insG*
BF-20	L4.8	T1; T2; T3: T5	RIF+INH+EMB	*rpoB_S450S/C*+katG_S315T+ embB_M306V*
BF-21	L3.1.1	CAS1-kili	RIF+INH+EMB+STM	*rpoB_H445D +katG_S315T+ embB_M306I+ gid_c.386_386del, rrs_A514C*
BF-24	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+FLQ+ETO**	*rpoB_S450L+katG_S315T+embB_M306V+gyrA_D94A,gyrA_A90V+ethA_M1R*
BF-26	L2.2.1	Beijing	RIF+INH	*rpoB_D435V +katG_S315T*
BF37	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+FLQ+ETO**	*rpoB_H445C + katG_S315T + embA_c.-16C>T, embB_M306I+ gyrA_D94G+ ethA_M1R*
BF-38	L4.2.2	T;LAM7-TUR	RIF+INH+STM	*rpoB_S450L +katGS315T + rrs_A514C*
BF-39	L2.2.1	Beijing	RIF+INH	*rpoB_D435V + katG_S315T*
BF-40	L4.2.2	T;LAM7-TUR	RIF+INH	*rpoB_S450L + katG_S315T*
BF-42	L4.2.2	T;LAM7-TUR	RIF+INH+PZA+EMB	*rpoB_S450L +katG_S315T+ pncA_T135I+ embB_H1002R*

BF-43	L2.2.1	Beijing	RIF+INH+PZA+ EMB+ STM	*rpoB_D435V +katG_S315T+ pncA_V139G+ embB_M306V + rrs_C517T*
BF-44	L4.2.2	T;LAM7-TUR	RIF+INH	*rpoB_H445C + katG_S315T*
BF-45	L4.2.2	T;LAM7-TUR	RIF+INH+EMB+ PZA	*rpoB_S450L + katG_S315T+ embB_H1002R + pncA_T135I*
BF-58	L3.1.1	CAS1-kili	RIF+INH+PZA+EMB+STM+ FLQ+ETO+CAP**	*rpoB_H445G+katG_S315T+pncA_L27P+embB_M306I, embB_G406S+gid_c.386_386del+rrs_A514C+gyrA_D94N+ ethA_c.11A>G+ tlyA_c.358_359insG*
BF-60	L2.2.1	Beijing	RIF+INH	*rpoB_D435V + katG_S315T*

*Mixed base; RIF=Rifampicin; INH=Isoniazid; PZA=Pyrazinamide; EMB=Ethambutol; FLQ=Fluoroquinolones (Levofloxacin, Ofloxacin, moxifloxacin); ETH=Ethionamide; STM=Streptomycin; CAP=Capreomycin; **Pre-XDR

Appendix 6: Similarity matrix displaying core-allele differences of the 15 MDR-MTBC strains from East Ethiopia. Alleles differences between strains ranged from 2 to 588.

	BF38	**BF24**	**BF16**	**BF12**	**BF3**	**BF15**	**BF1**	**BF20**	**BF21**	**BF26**	**BF43**	**BF58**	**BF45**	**BF60**	**BF8**
BF38	0	49	45	571	567	559	43	392	570	541	558	570	41	559	562
BF24	49	0	50	587	581	575	48	406	587	557	581	585	47	583	577
BF16	45	50	0	584	579	572	5	402	584	554	577	582	44	580	573
BF12	571	587	584	0	22	44	581	585	24	562	579	2	574	583	22
BF3	567	581	579	22	0	38	576	581	22	557	573	21	570	577	16
BF15	559	575	572	44	38	0	569	576	43	550	568	43	565	572	33
BF1	43	48	5	581	576	569	0	400	582	553	577	580	42	578	572
BF20	392	406	402	585	581	576	400	0	585	561	585	585	401	588	579
BF21	570	587	584	24	22	43	582	585	0	559	580	23	577	582	22
BF26	541	557	554	562	557	550	553	561	559	0	6	561	552	2	554
BF43	558	581	577	579	573	568	577	585	580	6	0	578	575	6	571
BF58	570	585	582	2	21	43	580	585	23	561	578	0	573	581	21
BF45	41	47	44	574	570	565	42	401	577	552	575	573	0	576	567
BF60	559	583	580	583	577	572	578	588	582	2	6	581	576	0	574
BF8	562	577	573	22	16	33	572	579	22	554	571	21	567	574	0