Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

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Molecular epidemiology of multidrugresistant tuberculosis among pulmonary tuberculosis patients in Tigray Region,Ethiopia

Molekylær epidemiologi blant pasienter med multiresistent tuberkulose i Tigray, Etiopia

Letemichael Negash Welekidan

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Dedication

To

My Father!

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List of abbreviation and acronyms

AFB	acid-fast bacilli
AM	amikacin
CAP	capreomycin
DR-TB	drug-resistant tuberculosis
DST	drug-susceptibility testing
EPHI	Ethiopian Public Health Institute
FLQs	fluoroquinolones
FMOH	Federal Ministry of Health of Ethiopia
INH	isoniazid
KM	kanamycin
LPA	line probe assay
LTBI	latent tuberculosis infection
MDR-TB	multidrug resistant tuberculosis
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NAATs	nucleic acid amplification tests
NPV	negative predictive value
PCR	polymerase chain reaction
PPV	positive predictive value
Pre-XDR-TB	pre-extensively drug-resistant tuberculosis
PTB	pulmonary tuberculosis
RIF	rifampicin
RR	rifampicin resistance

SNPs	single nucleotide polymorphisms
TDR	totally drug-resistant
WGS	whole-genome sequencing
WHO	World Health Organization

Summary

Tuberculosis (TB), which is caused by closely related *Mycobacterium tuberculosis* complex (MTBC) species, is an ancient human disease that gravely affects millions of people every year. TB is a preventable and treatable infectious disease. The continuing emergence and spread of multidrug-resistant tuberculosis (MDR-TB) threaten the global TB control efforts. TB is the first killer among infectious diseases worldwide. According to the WHO report, there were an estimated 10.0 million incident cases, 1.4 million TB deaths, with more than 95% of these deaths in developing countries in 2019. Ethiopia is among the three high TB, TB/HIV, and MDR-TB burden countries. In 2019 in Ethiopia, there were 157,000 new TB cases, 1,400 MDR/RR-TB cases and 23,800 death from TB. This thesis aimed to describe the molecular epidemiology of multidrug-resistant *Mycobacterium tuberculosis* among pulmonary TB patients in Tigray Region, Ethiopia.

Three hundred sputum samples were collected from six hospitals of the Tigray Region between July 2018 and August 2019. The 227 samples culture positive for MTBC were subjected to drug susceptibility test to 1st- and 2nd- line anti-TB drugs using line probe assay. Among the 227 positive cultures, 74 samples were sequenced using whole-genome sequencing (WGS).

WGS analysis showed diversified *Mycobacterium tuberculosis* genotypes circulating in the region, with L4 as the predominant genotype. The overall proportion of MDR-TB was high. The high proportion of MDR-TB among new and previously treated patients is alarming and calls for an urgent intervention to improve patient management. The high proportion of MDR-TB among newly diagnosed cases and the high level of recent transmission index indicates an ongoing transmission, which suggests the need for an enhanced TB control program performance to interrupt transmission. The study highlighted the usefulness of mutations at *rpoB*, *katG*, *embB*, *rps*L, *pncA*, *ethA*, *gyr*A and *rrs* genes as a molecular marker for the rapid detection of resistance to RIF, INH, ETB, SM, PZA, ETH, FLQs and injectable 2nd-line anti-TB drugs, respectively. Besides the canonical mutations, a significant number of disputed *rpoB* mutations were also reported.

Overall, the regional TB control program should be strengthened to detect and provide appropriate early treatment and follow-up for drug-resistant TB (DR-TB) cases. Abiding by the five WHO-recommended priority actions for DR-TB management is necessary to reduce the current high MDR-TB burden in the study region. Periodic surveillance of drug-resistance conferring

mutations, early diagnosis and treatment of TB, and scaling up of drug susceptibility testing facilities to prevent and control the transmission of DR-TB in the community is recommended.

Sammendrag

Tuberkulose (TB) forårsakes av nært beslektede arter innen *Mycobacterium tuberculosis*-komplekset (MTBC), og er en eldgammel, alvorlig sykdom hos mennesker som rammer millioner hvert år. TB er en smittsom sykdom som kan forebygges og behandles, men fremveksten og spredningen av multiresistent tuberkulose (MDR-TB) truer den globale bekjempelsesinnsatsen. TB er den smittsomme sykdommen som gir størst antall dødsfall i verden. WHO anslår at det i 2019 var anslagsvis 10,0 millioner tilfeller, 1,4 millioner TB dødsfall, med mer enn 95% av disse dødsfallene i utviklingsland. Etiopia er blant de tre land som er mest belastet, med høye tall av TB, TB / HIV og MDR-TB. I Etiopia var det i 2019 157.000 nye TB-tilfeller, 1400 MDR tilfeller og 23 800 dødsfall fra TB. Denne doktorgradens overordnede mål var å beskrive den molekylære epidemiologien til sjukdom forårsaket av multiresistent *Mycobacterium tuberculosis* blant lungepasienter i Tigray-regionen, Etiopia.

Tre hundre sputumprøver ble samlet inn fra seks sykehus i Tigray-regionen mellom juli 2018 og august 2019. Av disse ble de 227 prøvene som var positive for MTBC, undersøkt med en følsomhetstest mot 1. og 2. linje anti-TB medisiner ved bruk av Line Probe Assay. Av de 227 positive kulturene ble 74 prøver sekvensert ved bruk av helgenomsekvensering (WGS).

WGS-analysene viste at forskjellige MTBC-genotyper som sirkulerte i regionen, med L4 som den dominerende genotypen. Den høye andelen MDR-TB blant nye og tidligere behandlede pasienter er alarmerende og stiller store krav til en forbedret pasienthåndtering. Den høye andelen MDR-TB blant nylig diagnostiserte tilfeller og det høye nivået av nylig overførte infeksjoner indikerer en pågående overføring, og antyder behovet for en forbedret ytelse av TB-kontrollprogrammet for å avbryte overføringen. Studien fremhevet nytten av mutasjoner ved *rpoB, katG, embB, rpsL, pncA, ethA, gyrA* og *rrs* gener som en molekylær markør for rask påvisning av resistens mot *RIF, INH, ETB, SM, PZA, ETH, FLQ* og injiserbare andrelinje anti-TB medisiner.

Samlet sett bør det regionale TB-kontrollprogrammet styrkes for å oppdage og gi tilpasset passende behandling og oppfølging av TB-tilfeller. Å identifisere TB-kontrollprogrammets begrensninger i regionen, og følge de fem WHO-anbefalte prioriterte tiltakene for styring av DR-TB, er nødvendig for å redusere den nåværende høye MDR-TB-belastningen i studieområdet. Det anbefales periodisk overvåking av mutasjoner som gir antibiotikaresistens, tidlig diagnose og

behandling av TB, og oppskalering av fasiliteter for testing av isolater for antibiotikafølsomhet for å forhindre og kontrollere overføring av DR-TB i samfunnet.

List of papers

- **Paper 1:** Characteristics of Pulmonary Multidrug-Resistant Tuberculosis Patients in Tigray Region, Ethiopia: a cross-sectional study. Plos One August 14, 2020; *https://doi.org/10.1371/journal.pone.0236362*
- **Paper 2**: Frequency and Patterns of First- and Second-Line Drug Resistance-Conferring Mutations of *Mycobacterium tuberculosis* isolated from Pulmonary Tuberculosis Patients in a Cross-Sectional Study in Tigray Region, Ethiopia. Journal of Global Antimicrobial Resistance 3 December 2020; *https://doi.org/10.1016/j.jgar.2020.11.017*
- **Paper 3**: Whole-Genome Sequencing Revealed Disputed *rpo*B Mutations and Lineage-Specific Association with Drug-Resistance of *Mycobacterium tuberculosis* in Tigray Region, Ethiopia. Manuscript.

General introduction

Tuberculosis: Historical perspective

TB is caused by bacteria belonging to the MTBC and has plagued human and animal populations for millennia (1). According to some scientists, the genus *Mycobacterium* could exist 150 million years ago (2), and the early progenitor of *Mycobacterium tuberculosis* (Mtb) may have existed in East Africa as early as 3 million years ago. Mtb's long-term existence lead scientists to suspect the infection might have started during the early hominids period (2). However, modern Mtb strains' common ancestor might have emerged 20.000-15.000 years ago (3).

The first written documents about TB originate in India as early as 3300 years ago and in China 2300 years ago (4,5). During the ancient time when the term TB was unknown, different countries used to name it differently. In ancient Greece, the term "*phthisis*", in ancient Rome "*tabes*" and ancient Hebrew "*schachepheth*" was used. In the 1700s, TB was called "*the white plague*" due to the patients' paleness. Johann Schonlein coined the term "tuberculosis" for the first time in 1834 (6). In 1865 Jean-Antoine Villepin showed TB to be contagious, and the causative agent of TB, the tubercle bacillus, was discovered by the prominent scientist Robert Koch. He disclosed his findings to the Society of Physiology in Berlin on 24th March 1882 (3).

The description of TB and its causative agent leads to the massive wave of treatment facilities like TB sanatoria being the first in the USA in 1884, and the modern era of discovery of anti-TB drugs like streptomycin (SM) in 1944 and isoniazid (INH) in 1952 (**Figure 1**) (2).

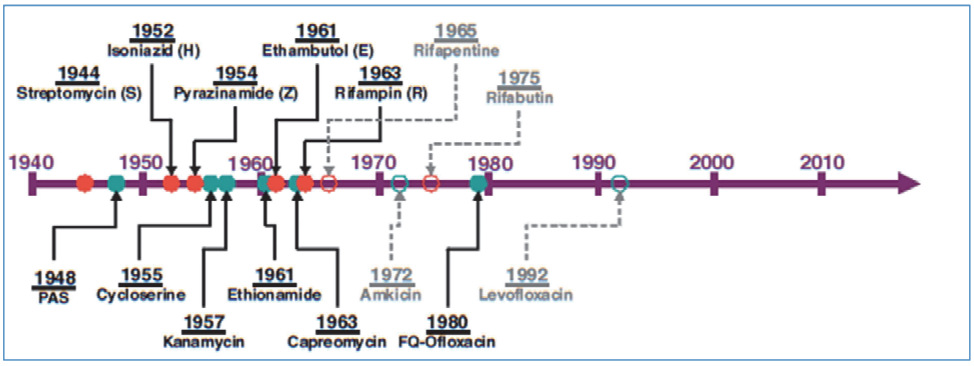

Figure 1. The modern history of the development of anti-TB drugs. With permission (9)

Mycobacterium tuberculosis complex (MTBC)

Mycobacterium, the only genus in the family of *Mycobacteriaceae,* is a diverse group of bacteria. Mycobacteria are non-motile, non-spore-forming, aerobic and gram-positive rods that are acid-fast due to the presence of medium to long chains of mycolic acids in their cell wall. They have a thick, lipid-rich and hydrophobic cell wall. The cell wall's thickness and composition ensure that mycobacteria are impermeable to hydrophilic nutrients and resistant to heavy metals, disinfectants, and antibiotics. The genus *Mycobacterium* encompasses more than 170 species with a diversified ability to cause diseases in humans. Mycobacteria belonging to the MTBC are closely related, with a 99.9% DNA sequence similarity. The most prominent member of the MTBC is Mtb, the leading cause of TB in humans (1,7).

Based on the difference in epidemiology, disease and growth in vitro, the genus *Mycobacterium* is divided into four groups. These are MTBC (the cause of TB), *M. leprae*, *M. ulcerans* and nontuberculous mycobacteria (NTM). The genus-species includes strict pathogens, potential or opportunistic pathogens, and nonpathogenic (saprophytic species) (8). Details on members of MTBC are shown in **Table 1**.

Table 1: Members of MTBC (9,10)

MTBC species	**Primary host**	**Mode of transmission to humans**	**Disease in humans**
Mycobacterium tuberculosis	Human	Inhalation of bacilli	TB
M. africanum	Human	Inhalation of bacilli	TB
M. bovis	Bovine (cattle, bison, and deer perpetuate)	Drinking unpasteurized milk, eating infected animals' meat, contact with infected animals' saliva, urine, or droppings, and discharging lesions or in rare cases via inhalation	TB
M. bovis BCG	-	-	-

Mycobacterium caprae	lesions from cattle, pigs, red deer (*Cervus elaphus*), and wild boars	Contact with TB lesions	TB
M. canettii	Small rodents	-	TB
M. microti	Vole (rodents)	-	TB
M. pinnipedii	Seals and sea lions	-	TB
M. mungi	Mongoose	-	TB
M. orygis	Oryxes and Bovidae	-	TB
Mycobacterium suricattae	Meerkats	-	TB
Mycobacterium dassie	Rock hyraxes	-	TB

Lineages of *Mycobacterium tuberculosis*

A coalescent analysis revealed the emergence of MTBC about 70 thousand years ago. The migration of MTBC followed the modern human movement out of Africa and expanded with increasing human population density during the Neolithic ages (11).

Moreover, the increased TB mortality in Africa was driven by introducing European strains of MTBC and the expansion of selected native strains having a fitness benefit in post-colonial Africa's urbanized settings (12). Current genomic data show that the MTBC encompasses five human-adapted lineages, including Mtb sensu stricto (L1–4 and L7), two additional human-adapted lineages referred to as *M. africanum* (L5 and L6), and at least nine animal-adapted lineages (13). Furthermore, recently discovered lineages of MTBC are claimed to be restricted to the African Great Lakes region (L8) (14) and East of Africa (L9) (15).

In Ethiopia, a systematic review on genotyping studies indicated the presence of several lineages, including Indo-Ocean (L1), East Asian/Beijing (L2), East African-Indian (L3), Euro-American (L4) and Ethiopian (L7). Amazingly, the Ethiopian lineage seemed to be localized to the northeastern part of Ethiopia (16). The various MTBC genotypes may have entered Ethiopia through human movement, trade, migration, and tourism (17).

Tuberculosis transmission and symptoms

TB mainly spreads through the inhalation of infectious aerosols by close person-to-person contact when a person with active TB coughs, sneezes, or talks in close proximity to another susceptible individual. Large particles are trapped on mucosal surfaces and removed by the ciliary activity of the respiratory tract. However, small particles containing one to three tubercle bacilli can reach the alveolar spaces and establish infection. Though TB can cause disease in almost any part of the body, most infections in immune-competent patients are restricted to the lungs (pulmonary TB) and are characterized by tubercle formation from which the name TB is given. If not affecting the lungs, the term extrapulmonary TB is often used (18).

Exposure to TB bacilli and infection progression depends on several factors like the TB patient's infectiousness, the environment in which the exposure occurred, frequency and duration of the exposed individual and susceptibility (immune status) of the exposed person. Household members, friends, roommates, and co-workers who spend a long time in a closed environment with infectious TB disease are highly likely to be infected with MTBC **(Figure 2)** (20).

Although there is some chance of TB transmission outdoors, almost all transmissions occur more successfully indoors, like in homes and congregate settings such as hospitals, clinics, refugee camps, factories, shelters for homeless, prisons, and most public transportations (20–22). Hence the best way of TB transmission interruption is to isolate the index cases and treat them appropriately (19). TB contacts are at high risk of infection. They should be investigated properly to identify active TB early and reduce transmission of the disease to the community. Identification of latent TB infection (LTBI) is also imperative to allow preventive measures (23).

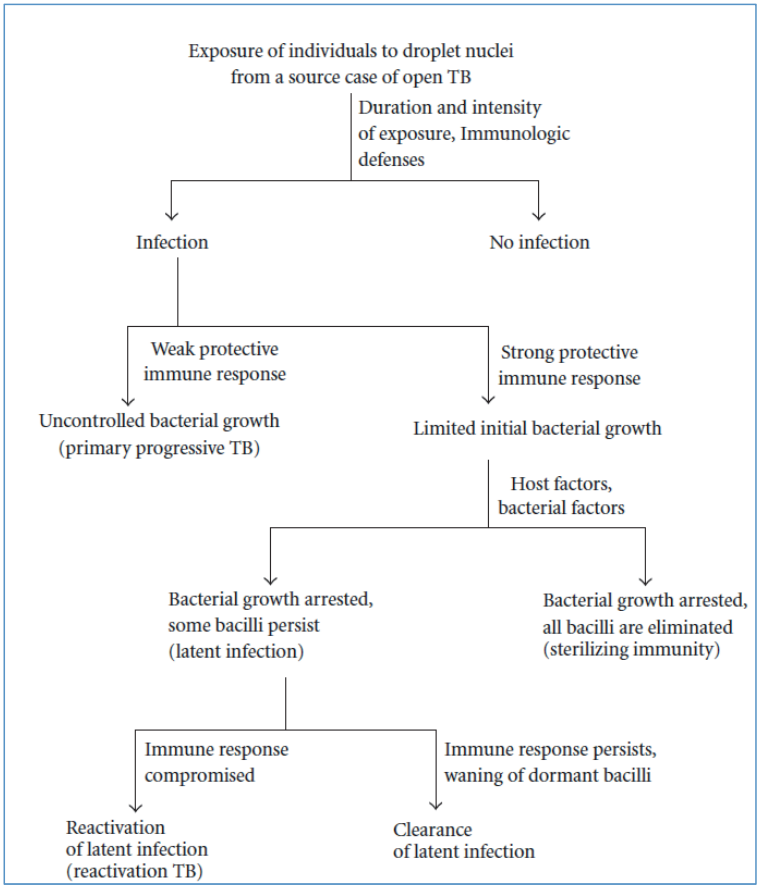

Figure 2: Progression and outcome of immunocompetent individuals following exposure to MTBC droplet nuclei transmitted from sputum smear-positive pulmonary TB. With permission (24)

Pulmonary TB (PTB) disease symptoms include a dry or productive cough that lasts two weeks or longer, pain in the chest, coughing up blood (hemoptysis), weakness or fatigue, weight loss, appetite loss, chills, fever and night sweating (25).

Diagnosis of TB and detection of DR-TB

Despite success in improving cure rates for TB, diagnosis and early case detection remain a significant obstacle to TB control programs (26). The main concerns of the global TB prevention and control strategies are the rapid, accurate and early detection and treatment of TB cases. The main focus is on smear-positive TB patients which are the most infectious form of TB cases. Early

detection of smear-negative TB, commonly identified among human immunodeficiency virus (HIV) positive persons, children, and MDR-TB cases, is crucial (27,28).

The alarming increase of MDR and extensively drug-resistant tuberculosis (XDR-TB), mainly associated with HIV co-infection, intensifies the challenge of diagnosing and treating TB. The limitation of conventional diagnostic techniques is often a misdiagnosis of HIV-associated TB. Hence, the increasing transmission and death of MDR/ XDR-TB patients co-infected with HIV have highlighted the urgent need for rapid diagnostic methods. There is no single absolute diagnostic technique that fulfills all the demands; rapid, affordable, and user-friendly (27).

Rapid progress in the speed and quality of TB diagnostic services like liquid culture systems and molecular LPAs to detect MDR-TB has been observed in the last decades. However, the complexity, cost, need for sophisticated laboratory infrastructure and trained personnel limited their use due to resource constraint in many high TB burden countries (27,28). The Ministries of Health in the respective countries should choose the most suitable and reliable tests for detecting TB disease based on their national strategic plans for laboratory strengthening and the context for testing, test availability, and overall cost-effectiveness (9). The TB diagnostic techniques include the chest X-ray, acid-fast bacilli (AFB) smear microscopy, mycobacterial culture, nucleic acid amplification tests and immunological methods (29).

Smear microscopy

Smear microscopy remains the most widely used method for diagnosing TB in developing countries and is suitable for peripheral and higher-level laboratories. It is simple, inexpensive, detects TB relatively rapidly, and is necessary for treatment follow-up of patients with susceptible TB in a low-level laboratory situation to prevent laboratory-acquired infections. For many years, WHO and the International Union against Tuberculosis and Lung Disease (IUATLD) recommended sputum smear microscopy as the cornerstone to diagnose PTB, as smear-positive subjects are most contagious (9,30,31). According to the recommendation of IUATLD, the use of three sputum specimens (spot-morning-spot) for the diagnosis of TB cases and a single examination of "morning" sputum specimens on three occasions for follow-up of treatment. The occasion for the collection of the sputum is one at the end of the intensive phase (end of 2nd-month), one during the continuation phase (end of 5th-month), and another one at the end of treatment (end of 6th-month) (29). However, the recommended number of sputum specimens required for

examination using microscopy has been changed from three samples to two samples (spot-spot) (32). The one sputum sample is adequate to identify the majority (95-98%) of smear-positive TB patients, given that the settings have an appropriate external quality assessment and documented good-quality microscopy (9).

The drawback of smear microscopy for detecting TB is that it is relatively insensitive, with a detection limit of 5000–10,000 organisms/mL compared to 10–100 organisms/ mL for culture and specimens with < 5000 bacteria negative by microscopy. The sensitivity of smear microscopy is 55.7% (33) and is further reduced in patients with extrapulmonary TB, children and patients co-infected with HIV. It cannot distinguish MTBC from NTM, viable from nonviable organisms and drug-susceptible strains from DR strains (9). Therefore, a systematic review suggested the need for performing smear microscopy and mycobacterial culture tests for presumptive TB cases (34).

Mycobacterial culture

The introduction of microbial culture has significantly improved TB diagnosis compared to microscopy, mainly in high TB burden countries (30). Mycobacteria can be grown in specific solid or liquid media, and its growth can be visually identified by the specific growth characteristics or by automated detection of its metabolism (9). Mycobacterial culture is highly sensitive and can detect 10 to 100 viable bacilli per ml of sputum. The sensitivity and specificity of solid culture are 77% and 93.4%, respectively, compared to liquid culture (35). It further allows species identification and drug susceptibility testing (DST) and can regulate treatment response for DR-TB patients (32). Mycobacterial culture is mainly done on solid media (the Lowenstein- Jensen slope) or in broth media. However, mycobacteria's growth on conventional media is slow and takes two to four weeks from the AFB-positive sample and four to eight weeks from the AFB-negative sample. The turnaround time (TAT) for DST results using the solid-based method ranges from eight to twelve weeks (36). This waiting time for the detection and DST contributes to increased transmission as those infected individuals continue to transmit DR-TB (18).

The utilization of liquid media using automated machines such as the BACTEC 320 reduced the time required for MTBC detection (37). In 2007, WHO recommended using liquid culture and DST in low- and middle-income countries (38). The liquid media based tests such as BACTEC® (BD Diagnostics, Sparks, MD, USA), MGIT® (BD Diagnostics), and BacT/ALERT® (bioMe' rieux SA, Marcy l'Etoile, France) have a shorter TAT that ranged from 10 to 14 days for

identification and additional 10 days for DST compared to four to six weeks with conventional solid media. Thus liquid media remains the gold standard for initial isolation through the half-automated radiometric BACTEC 460 TB system (BACTEC 460) (34,39). The sensitivity and specificity of the BACTEC 460 system for detecting mycobacteria were 99.6% and 99.9%, respectively. The use of liquid media increased mycobacteria's detecting rate by 10% compared to solid media, consequently improving patient management (34).

The radiometric BACTEC 460 TB system's application is restricted by the use of radioactive material, difficulties in manual loading and unloading, the potential hazard of needle stick injury, risk of cross-contamination, and lack of computerized data management.

The BACTEC Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT), a newly developed nonradiometric, fully automated, continuously monitoring system, was introduced as an alternative to the radiometric BACTEC 460 for detection of mycobacteria (39). The BACTEC 960/MGIT systems performance in detecting mycobacteria showed a sensitivity and specificity of 81.5% and 85.8%, respectively. The detection time was considerably shorter than the BACTEC 460 and solid media (39).

The drawbacks of the liquid media-based tests are: high cost, requiring specialized laboratories and trained laboratory personnel, result delay on mycobacterial species' identification and they are prone to contamination (5%–10%) (39). Solid media provide all this information with a simple observation of colonies (34). The limitation of traditional culture and DST services is the slow scaling up, expensive, long TAT, compounded by enormous demands on laboratory infrastructure and human resources (27).

Mycobacterial culture largely remains the gold-standard technique for DST. The current guideline recommends a combination of conventional solid media with a broth-based method to ensure purity and sufficient strength for the diagnosis of mycobacterial infection (27,32,34).

Nucleic-acid amplification tests (NAATs)

NAATs are designed to amplify the nucleic acid regions specific to MTBC (40). They have shown significant importance for rapid TB diagnosis, rapid detection of DR with standardized testing, high throughput potential, and fewer requirements for ensuring laboratory biosafety. Since the polymerase chain reaction (PCR) innovation in the early 1980s, genomic diagnostics have impacted clinical medicine (27). In 2008, WHO endorsed using NAATs for detecting DR to

rifampicin (RIF) alone or in combination with INH in resource-limited settings in response to the rising challenge of global DR epidemics (41,42). These tests include the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, United States) and LPAs, such as the GenoType MTBDR*plus assay* (Hain Life Science, Nehren, Germany), which detects the presence of mutations associated with drug resistance to RIF (42).

A meta-analysis and meta-regression review revealed that the pooled sensitivity and specificity for NAATs were 85% and 97%, respectively (43). A study report on the performance of NAATs showed the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) on specimens positive for AFB smear were 96.0%, 95.3%, 98.0%, and 90.9%, respectively. Besides, the sensitivity, specificity, PPV and NPV on specimens negative for AFB smear were 79.3%, 80.3%, 83.1%, and 76.0%, respectively (44). The majority of AFB smear-negative specimens are from people living with HIV. About 80% of the TB patients co-infected with HIV were reported from Sub-Saharan Africa, where there is a scarcity of laboratory diagnostics (41).

NAATs overcome the limitation of a long TAT of conventional solid-based methods to get DST results, ranging from 8 to 12 weeks (36). This delay plays a significant role in continuing the transmission of the DR-TB to the communities. Nucleic acid amplification methods provide tremendous advantages over conventional DST, including rapid diagnosis and standardized testing (18).

Despite the advantages of NAATs in the rapid diagnosis of TB and detection of DR, its use is mostly limited by the complexity of DNA extraction, amplification and detection, and the biosafety concerns related to manipulating MTBC. Moreover, NAATs have shown less sensitivity than microbiological culture, especially in smear-negative TB cases. Hence, conventional laboratory techniques (AFB smear microscopy, mycobacterial culture, and DST) must be maintained to monitor patient response to treatment and detect resistance to drugs other than RIF (27).

Line probe assay (LPA)

LPAs are rapid DNA-based tests that simultaneously confirm the presence of TB and detection of RIF-R (45). In 2008, WHO endorsed LPAs for detecting MTBC and rapid detection of RIF-R from sputum smear-positive and in cultured isolates of MTBC (41,42). The diagnostic performance of the two commercially available LPAs: the INNO-LiPA Rif.TB assay (Innogenetics, Ghent, Belgium) and the GenoType MTBDR*plus* (Hain version 1 (Hain Lifescience, Nehren, Germany))

showed excellent accuracy for the detection of RIF-R. However, the diagnostic accuracy for INH-R showed lower sensitivity, despite excellent specificity. The recommendation was limited to culture isolates or smear-positive sputum specimens of pulmonary and extrapulmonary samples due to inadequate data to stratify based on smear status's diagnostic accuracy (42).

In November 2015, WHO recommended to use the two newly developed LPAs (Hain GenoType MTBDRplusV2 and Nipro NTM+MDRTB (Tokyo, Japan)). The sensitivity and specificity of Hain V1, Hain V2, and Nipro to detect isolates were 90.3%/98.5%, 90.3%/98.5%, and 92.0%/98.5% for detection of RIF-R, and 89.1%/99.4%, 89.1%/99.4%, and 89.6%/100.0% for detection of INH-R, respectively comparing to phenotypic DST and targeted sequencing. The sensitivity and specificity for testing of sputa were 97.1%/97.1%, 98.2%/97.8%, and 96.5%/97.5% for RIF-R and 94.4%/96.4%, 95.4%/98.8%, and 94.9%/97.6% for INH-R, respectively comparing to the phenotypic DST (46). Results were similar for direct and indirect MTBC detection and across LPAs with a pooled sensitivity of 94% for smear-positive specimens and 44% for smear-negative specimens (47).

The unavailability of rapid molecular tests for detecting XDR-TB in areas with high MDR rates and XDR-TB poses a serious threat to effective TB infection control and adequate patient management globally, especially where there is a limited DST capacity. The GenoType MTBDR*sl* test is a PCR-based amplification and reverse blotting novel assay that uses specific probes hybridized to nitrocellulose strips to detect resistance to 2nd-line drugs and ethambutol (EMB) (48). The MDR-TB detection test showed high specificity (95.8 to 100%) compared to that of conventional agar proportion DST. The sensitivities of GenoType MTBDR*sl* (v1.0) were 85.1%, 56.2%, 43.2%, 84.2%, and 71.4%, for the detection of FLQs, EMB, kanamycin (KM), amikacin (AM) and capreomycin (CAP) resistances, respectively comparing with the conventional DST (48).

The Genotype MTBDR*sl* (v2.0) assay, which includes probes for detection of mutations in the e*is* promoter region, showed better performance accuracy than v1.0 for the detection of XDR-TB, with high specificity and sensitivities of 81.8% and 80.4% for direct and indirect testing, respectively compared to phenotypic DST and sequencing. Thus, MTBDR*sl* v2.0 represents a reliable test for the rapid detection of resistance to 2nd-line drugs and a useful screening tool to guide appropriate MDR-TB treatment initiation (49).

The limitations of LPAs are that they are technically demanding, costly and restricted to centralized laboratories. They are not recommended for directly testing smear-negative clinical specimens because of limited sensitivity and risk of cross-contamination. Scale-up and impact of these assays have, therefore, been limited (41).

GeneXpert MTB/RIF assay

The innovation of Xpert MTB/RIF assay overcomes several limitations observed by LPA. In December 2010, WHO endorsed the use of Xpert MTB/RIF assay (Cepheid Inc., Sunnyvale, CA, United States) as the initial diagnostic test in individuals suspected of having MDR-TB or HIV associated TB in adults and children. Besides, as a follow-on to smear microscopy in settings where MDR-TB and HIV-associated TB were of less concern (13). These recommendations were updated in October 2013 to expand its use as the initial diagnostic test in all persons (adults and children) with signs and symptoms of TB (50).

Summary of WHO's 2013 policy recommendations on using Xpert MTB/RIF to diagnose pulmonary TB and RIF resistance in adults and children (27)**:**

- Xpert MTB/RIF should be used rather than conventional microscopy, culture, and DST as the initial diagnostic test in adults suspected of having MDR-TB or HIV-associated TB (strong recommendation, high-quality evidence).
- Xpert MTB/RIF should be used rather than conventional microscopy, culture, and DST as the initial diagnostic test in children suspected of having MDR-TB or HIV-associated TB (strong recommendation, very low-quality evidence).
- Xpert MTB/RIF may be used rather than conventional microscopy and culture as the initial diagnostic test in all adults suspected of having TB (conditional recommendation acknowledging resource implications, high-quality evidence).
- Xpert MTB/RIF may be used rather than conventional microscopy and culture as the initial diagnostic test in all children suspected of having TB (conditional recommendation acknowledging resource implications, very low-quality evidence).
- Xpert MTB/RIF may be used as a follow-on test to microscopy in adults suspected of having TB and who are not at risk of MDR-TB or HIV-associated TB, especially when

further testing smear-negative specimens is necessary (conditional recommendation acknowledging resource implications, high-quality evidence).

The assay is the only self-contained cartridge-based, fully automated DNA testing platform that can accurately detect both MTBC and RIF-R simultaneously in less than 2 hours. It is the only mature hemi-nested real-time PCR (rt-PCR) among the new generation automated molecular diagnostic platforms and the first robust and straightforward molecular test to be used outside conventional laboratory settings (27,41). The assay uses three specific primers and five unique molecular beacon probes (A-E) (13) directly on sputum, processed sputum sediment, and selected extrapulmonary specimens from both adults and children (50).

The positivity rates for microscopy, GeneXpert MTB/RIF and culture were 18.9%, 23.9%, and 20.4% for pulmonary samples and 9.7%, 18.4%, and 12.8% for extrapulmonary samples, respectively. GeneXpert assay showed almost the same performance for pulmonary and extrapulmonary samples with a sensitivity and specificity of 78.2% and 90.4%, and 79.3% and 90.3%, respectively. The assay detected 11% more TB cases than culture and 27% more cases than AFB microscopy (51). The sensitivity and specificity of GeneXpert for detection of RIF-resistance were reported to be 71% and 100%, respectively, compared with the standard proportional method (52).

GeneXpert assay's advantages are that they provide results quickly and need minimal technical training to run the test. RIF-resistance is a surrogate marker of MDR-TB because mostly RIF-resistance co-exists with resistance to INH. Therefore, RIF-R's rapid detection greatly helped patients initiate treatment much faster than the other DST techniques (53). The method's simplicity makes the assay widely implemented outside the centralized laboratories that can play an important role in TB control programs (41). The concern is to store cartridges in high-volume settings that can face a shortage of enough space. Besides, the waste generated by this assay is considerably higher than that of microscopy, and it requires a continuous and stable electrical power supply and annual calibration of the modules, which may pose problems in remote settings (27).

Chest radiography

Though chest x-ray is not a specific test, as TB symptoms and atypical radiologic findings are indistinguishable from those of community-acquired pneumonia, it is still useful for diagnosing TB due to the low sensitivity of sputum smear microscopy in diagnosing TB patients living with

HIV (34,54). Hence, in 2007, WHO recommended chest X-ray to support the PTB diagnosis for patients living with HIV and are sputum smear microscopy negative (54). In 2016, the WHO announced new recommendations and guidelines on chest radiography usage for PTB diagnosis in the national TB care program (55). Chest radiography can be used as a sensitive tool in diagnosing childhood pulmonary and extrapulmonary TB and excluding active TB before treating LTBI. It is also used as a sensitive tool for screening active TB to reduce the number of patients who need further definite diagnostic tests (56) and also used in TB prevalence surveys (57). However, its wider use is limited due to unavailability at peripheral health setting and the difficulty of results interpretation (58).

Immunodiagnostic tests of TB infection

The two commercially available immunological tests are tuberculin skin test (TST) and interferon-gamma release assay (IGRAs) to determine MTBC infection, mainly in individuals where direct detection of the organism is difficult (59).

TB skin test for diagnosis of latent TB infection

LTBI is a state of a persistent immune response to MTBC antigens' stimulation without active TB clinical manifestations. About 5-10% of individuals with LTBI can get TB in their lifetime (59). One of the important mechanism of preventing the development and transmission of active TB in the community is diagnosing and providing prophylactic treatment for LTBI (18).

The TST was developed in the 19th century (60). In 2015, the WHO recommended TST for diagnosing LTBI in high- and upper medium-income countries with low TB burden. The recommendation includes patients with HIV, adult and child contacts of PTB cases, patients initiating anti-tumor necrosis factor treatment, patients receiving dialysis, patients preparing for organ or hematologic transplantation, and patients with silicosis (61). TST limitations are the inability to differentiate between active TB and LTBI, previous infection and LTBI, false-positive tests due to NTM infection and false-positive tests due to Bacillus Calmette Guerin (BCG) vaccination (62). Other limitations include; incorrect method of TST administration, incorrect interpretation of reaction, an incorrect bottle of antigen used, false-negative reactions due to cutaneous anergy, recent TB infection (eight-ten weeks), old TB infection, very young age (less than six months old), recent live-virus vaccination/viral illnesses (e.g., measles and smallpox) and

overwhelming TB disease (63,64). A sputum culture should be done as a confirmatory test to rule out an active TB disease (18).

Interferon-gamma release assays (IGRAs) for diagnosis of latent TB infection

The two commercially available IGRAs are Quanti FERON-TB Gold (QFT; Cellectis/Qiagen, Carnegie, Australia) and T-SPOT.TB (T-SPOT; Oxford Immunotec, Abingdon, United Kingdom). IGRAs measure Interferon-γ (IFN-γ) secreted by the patient's T-lymphocytes (QFT) or the number of IFN-γ secreting lymphocytes (T-SPOT) following stimulation of the cells with MTBC-specific antigens that are not found in BCG vaccine strains or most NTM species (59).

IGRAs are not commonly used in resource-limited settings due to the requirement for advanced technical expertise and expensive equipment (18). In 2015, WHO recommended using IGRAs to diagnose and treat LTBI in high- and upper medium-income countries with low TB burden. The recommendation is for HIV-infected patients, adult and child contacts of PTB cases, patients initiating anti-tumor necrosis factor treatment, patients receiving dialysis, patients preparing for organ or hematologic transplantation, and silicosis patients. Prisoners, health care workers, immigrants from high TB burden countries, homeless persons and illicit drug users are among the recommended individuals (61).

The advantages of the IGRAs are that the assay needs a one-time patient visit to perform the test. Results can be collected within 24 hours and BCG vaccination does not result in a false-positive result. However, disadvantages include the requirement of a short time to process the collected blood specimen, require expensive laboratory facilities, and the assays are used only to test for LTBI. Additionally, IGRAs may not provide accurate results in people who are HIV- positive (65).

Fine needle aspiration cytology (FNAC)

The conventional methods used to diagnose extrapulmonary TB are smear microscopy, histopathology and culture. Though rapid molecular tests revolutionize the continuing use, FNA was the crucial method with a minimally invasive technique to diagnose peripheral lymphadenopathy for nearly two decades. FNAC is simple, economical and helps in diagnosing tuberculous lesions (66).

Extrapulmonary TB diagnosing accuracy of FNAC revealed that the sensitivity, specificity, PPV, and NPV were 79.7%, 48.1%, 31.9%, and 88.6%, respectively, with a poor agreement (Kappa test= 0.18) with culture. However, the sensitivity, specificity, PPV and NPV for Xpert were 95.9%,

60.5%, 42.6% and 98%, respectively with a slight agreement (Kappa tes= 0.39) with culture. Thus, the Gene Xpert is an accurate test to diagnose extrapulmonary TB where there is no granuloma or caseation. In contrast, cytomorphology is suitable for diagnosing granulomatous and reactive, and other malignancies (67). FNAC's limitations include the occurrence of a technical and interpretative error during diagnosis, inadequate quantity of samples in small pulmonary lesions, or fibrosis (66).

Lateral flow urine lipoarabinomannan assay (LF-LAM)

One of the significant challenges for TB care and control is early and improved case-detection of smear-negative cases, often associated with HIV co-infected cases and children (68). In 2015-2016, WHO recommended the use of urine lateral flow LAM assay (LF-LAM) strip-test through the Alere Determine™ TB LAM Ag, United States of America (USA) (AlereLAM), the only commercially available urinary LAM test (69,70).

Determine™ TB LAM Ag test (LF-LAM) is a urine test for detecting LAM antigen, a lipopolysaccharide present in mycobacterial cell walls, released from metabolically active or degenerating bacterial cells and mainly found in people with active TB disease (68). This test is a potential point-of-care test for TB diagnosis only in HIV-positive adults and children with suggestive TB who have a CD4 cell count ≤100 cells/μL, or HIV-positive patients who are seriously ill regardless of CD4 cell count or with unknown CD4 cell count. Unlike the traditional diagnostic methods, urinary LAM assays showed a high sensitivity for TB diagnosis among individuals co-infected with HIV, even greater sensitivity in patients with lower CD4 cell counts. As the LAM assay has suboptimal sensitivity, it is not suitable for diagnosing TB for all populations with suggestive TB (70).

Drug susceptibility testing

A reliable DST for anti-TB drugs is imperative for adequately managing DR-TB patients and preventing further DR-TB evolution in these patients (71). The indirect methods, absolute-concentration method, resistance ratio method, and proportion methods were widely used methods for performing DST to INH, SM, and para-aminosalicylic acid (PAS), which were described by Canetti *et al.* in 1963 (72).

The DST methods are categorized into direct and indirect tests. In direct tests, the processed sputum samples or other pathological material are directly inoculated on the drug-containing

medium. In indirect tests, the drug-containing medium is inoculated from primary grown culture (73).

Absolute concentration method

The purpose of this method was initially to determine the minimum inhibitory concentrations (MICs) of INH and SM by the standard inoculum of MTBC to the drug-containing media and the control without the drug (72). The MICs are determined by preparing sequential two-fold dilutions of the drug to be tested and inoculated into the respective media. The lowest concentration of the drug that inhibited growth (less than 20 colonies at the end of 4 weeks) is considered the MIC (74).

The absolute concentration method's performances were 100% for INH, 96 to 100% for RIF, 91 to 100% for SM, and 85 to 100% for EMB with 100% reproducibility for all the four drugs (75). The test accuracy depends on the standardization of the inoculum size of the test and reference strains and the drug's critical concentration (73).

Resistance ratio method

This method is a modified form of the absolute concentration method developed to avoid differences in the MICs of a specific strain (72). The resistance ratio is obtained by dividing the MIC of the test strain by the wild type H37Rv in the same test protocol. The resistance ratio of two or less is considered drug-susceptible. However, eight or more are resistant and with rare intermediate DR strains. The test accuracy depends on the standardization of the inoculum size of the test strain and inoculum size of the wild reference strain (73,74).

Proportion method

This method has been implemented widely across the world since its innovation by the Pasteur Institute in Paris (73). The resistance can be determined by culturing from low and high serial dilutions of the inoculum. The ratio of colony-forming units between drug-containing and drug-free plates indicates the proportion of DR bacilli present in the bacterial population expressed in percentage. If the ratio is less than the critical proportion, the strain is considered susceptible, and if greater than the critical proportion, a strain is resistant (74). A study revealed no significant difference in the performance accuracy among the proportion method, Etest, and MGIT (76). However, the TAT to get DST results by the proportion method is longer than the methods mentioned above. Hence MGIT and Etest methods are easy and rapid, recommended for routine DST to replace the proportion method (76).

A comparison study between the proportion and resistance ratio methods for DST showed a high agreement between the two tests. The agreement rates were 93.2%, 93.7%, 93.2%, and 94.1% for INH, RFP, SM, and EMB, respectively (77). The advantage of the proportion method over the absolute concentration and resistance ratio methods are, accuracy does not depend on the standardization of inoculum size, controls are not necessary, and the ability to correlate critical drug concentrations and drug resistance proportions with clinical criteria (73,74).

Impregnated discs or strips (the Etest)

Wanger and Mills developed the epsilometer (Etest), an exponential gradient MIC technique, for DST of MTBC and other fastidious and slow-growing organisms (78). The Etest is a rectangular plastic strip impregnated with predefined series of two-fold dilutions of an antibiotic on one side of the strip from which the antibiotics diffuse into the media and a numeric scale that indicates the drug concentration on the other side of the strip. It is a quantitative method that dilutes and diffuses antibiotics into the medium and forms a diffusion gradient along the strip's length. The MIC value is when the growth inhibition ellipse intersects the MIC scale on the strip (79).

The test's performance accuracy agreed well with the results of agar proportion, radiometric BACTEC, and MGIT methods for DST of 1st- and 2nd-line anti-TB drugs (78,80). The categorical agreement between the agar proportion method and Etest was 100% for RIF, EMB, SM, and Ofx, and 98% for INH. The test has a TAT of 6 to 10 days (80). The method is cost-effective, easy, does not require expensive instruments and media, rapid and MIC values can be observed simultaneously, making it suitable for resource-limited countries instead of proportion (76,78–80).

Radiometric BACTEC 460TB method

The BACTEC 460 TB system, a semi-automated and radiometric technique, was introduced commercially in 1980 to identify MTBC from clinical samples and DST. The BACTEC 460 TB system's efficiency is based upon the use of liquid media with growth-promoting supplements that helped fast growth of isolates and helped to grow specific isolates, which cannot grow in solid media. BACTEC 460 TB system is far better than the solid media due to its short time for detection and DST, making it a gold standard for growth and DST (81).

The system is a modified form of the proportion method, which compares ^{14}C-labeled CO_2 (suggestion of growth) production by the standard amount of MTBC inoculum in a tube containing the required anti-TB drug and a tube without the ant-TB drug. Though it is a rapid test that needs

one week to get results, the system demands well-equipped laboratory infrastructure (including nuclear waste disposal). It is more expensive than the nonradiometric proportion method (74). The wide and continuing application of the BACTEC 460 TB System is limited by using a ^{14}C-labeled radioactive substrate to detect the CO_2 produced by microbial growth (81).

Automated nonradiometric Mycobacteria Growth Indicator Tube (MGIT) 960 system

DST using the MGIT 960 system is performed using an antibiotic susceptibility testing set, a growth control tube, and one tube for the respective drug, and a bar-coded tube carrier that holds the set. Together with the sample, a critical concentration of a drug is added to the MGIT tube, and then growth is compared between a drug-free control tube and a drug-containing tube. If the bacterial isolate is susceptible, growth is inhibited, and no fluorescence is produced in the drug-containing tube but presents in the drug-free tube. If the isolate is resistant to the particular critical concentration, growth occurred accompanied by fluorescence in the drug-containing tube and drug-free tube. The MGIT 960 system automatically indicates the growth and interprets DST patterns as susceptible or resistant. The isolate is categorized as resistant when 1% or more of the test population grows in the drug's critical concentration (71).

The accuracy of the direct MGIT 960 system for the detection of DR to RIF and INH were 97.0% and 94.1%, respectively, and for the indirect MGIT system was 98.0% for both drugs, taking the indirect method of proportion (MOP) as a gold standard (82). The MGIT system showed 96% agreement for detecting DR compared with proportion and resistance ratio methods (83).

The time required from specimen processing until DST results obtained ranged from 4 to 23 days for the direct MGIT method, 9 to 30 days for the indirect MGIT method and 26 to 101 days for the indirect MOP. Therefore, MGIT is reliable, fast, sensitive, efficient, and suitable for performing direct and indirect tests for early detection of MDR-TB in low-resource settings (82–84) and recommended to detect DST for 2nd-line anti-TB drugs (71).

A comparison study on the detection of DR to the critical concentration of SM, INH, or EMB using BACTEC MGIT 960 and the radiometric BACTEC 460 TB system showed an overall agreement of 96.7%, with a statistically insignificant difference among them. The test average TAT for BACTEC 460TB was 2.5 days shorter than BACTEC MGIT 960. Though the BACTEC 460 TB system had shown a slightly better detection of DR and shorter turnaround time, its use of ^{14}C-Labeled radioactive substrate made its application limited. Hence, BACTEC MGIT 960

appears to be a suitable replacement for the radiometric method of DST of MTBC (85). However, the wide implementation of MGIT is impeded by its high cost and prone to contamination (75,82,85).

The colorimetric redox-indicator methods

In this method, the growth of MTBC is determined by the principle that potassium nitrate (KNO_3) added into Löwenstein-Jensen medium (LJ) with or without drugs will be reduced into nitrite by the nitrate reductase enzyme of the bacteria. The reduction of nitrate to nitrite is evident in the color change of the indicator reagent added to the medium (86).

The performance of colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) test for INH and RIF by adding nitrate into broth cultures has shown a sensitivity and specificity of 100 and 95% for INH and 94 and 100% for RIF, respectively comparing with the BACTEC 460TB system as the "gold standard." The reproducibility of the test was excellent (for INH, kappa =1; for RIF, kappa =0.88) and short TAT for most isolates (5 days) (87). An evaluation study on the performance of nitrate reductase method using Löwenstein-Jensen medium revealed the sensitivities and specificities of 100 and 100% for RIF, 97 and 96% for INH, 95 and 83% for SM, and 75 and 98% for EMB, respectively, comparing with the BACTEC 460 method. Results were obtained in most isolates in 7 days.

The nitrate reductase method is rapid, accurate, and inexpensive for detecting MDR-TB, an alternative method that can replace the existing methods, such as the proportion and BACTEC methods, mainly in countries with resource constraints (86–88).

Microtiter based Alamar Blue (MABA) assay

The assay measures bacterial growth using fluorometric or colorimetric growth indicators (89,90). Alamar blue, a growth indicator soluble redox dye, is blue and nonfluorescent in the oxidized state. It turns into pink color and fluorescent in the reduced state following cell growth, which can be detected visually by a color change or a fluorometer. The assay has been used for the DST of anti-TB drugs in numerous laboratories (90). Besides, to assess the DST for the existing anti-TB drugs, MABA is used to determine the cytotoxicity or MIC of novel anti-TB drug candidates (89,91).

The overall MIC values for several anti-TB drugs obtained by visual/ fluorometric MABA were highly correlated with the BACTEC 460 system (92). The DR detection accuracy of MABA was better than the gold standard proportion method on solid medium (93). Thus, MABA is sensitive,

rapid, inexpensive, and nonradiometric and a potential alternative for screening and DST for anti-TB drugs (92,93).

In general, liquid media's efficiency and rapidity make it superior over the solid media for DST. In contrast, solid media are superior in checking the growing bacteria's colony morphology, determining the MIC of a drug, minimal contamination, and relatively low cost (75).

Molecular characterization of MTBC strains

MTBC strains are closely related to each other with >99% similarity of genome sequences though phenotypically different (94). Insertions/deletions/duplications and single nucleotide polymorphisms (SNPs) are a powerful tool for genomic variability and greatly influence MTBC's pathobiological phenotype (95). Differentiation of MTBC members is crucial for the proper management of TB patients and epidemiological purposes. The innovation of molecular typing has broadly disclosed various mycobacterial species' characteristics and helped fight the disease caused by the pathogens. MTBC characterization is used to identify misdiagnosed results, differentiate relapse from exogenous cases, trace the ongoing TB transmission chain, assess the relation between DR and a specific genotype, identify MTBC lineage distribution, and define the evolutionary features of MTBC (96).

The various genotyping methods for MTBC have their advantages and disadvantages. Since none of them are better than the other, the best typing method depends mostly on the sample under investigation, the setting where the typing is performed, and the research question (97). Some of the commonly used typing methods are presented below.

Regions of difference (RD) or deletion mapping and deligotyping

The present member of the MTBC strains evolved from a common ancestor through sequential deletion and insertion mechanisms. This insertion-deletion or region of difference (RD) is used to differentiate between MTBC and Mtb. RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, RD13, and RD14 are highly conserved in Mtb; hence help to differentiate Mtb from MTBC. Based on the presence or absence of Mtb specific deletion (TbD1), Mtb strains can be categorized into ancestral and modern strains (98).

RD-analyzer or deligotyping is a crucial and precise technique for identifying species, lineage, and sublineage prediction of MTBC using known RDs, also known as large-sequence polymorphisms

(LSPs), positioned in aggregations throughout the genome (99–101). Since the LSPs occurred because of significant inter-strain variability, they have been used as genetic markers for typing MTBC. Deletion analysis can be done either by a simple PCR-based technique or by automated microarray techniques (102). The latest version of the RD-analyzer is designed to allow user-defined RDs for lineage prediction (101). The technique's notion and protocol are designed based on the spoligotyping method, where deletions are detected in 43 genomic loci (102). The RD-analyzer's accuracy is 98% in species prediction with an agreement of 98.5% in Mtb lineage prediction compared to predictions based on single nucleotide polymorphism markers (101).

IS6110-restriction fragment length polymorphisms (RFLP) analysis

Mtb H37Rv reference strain WGS showed two main groups of repetitive DNA elements (tandem repeats (TR) and interspersed repeats (IR)) that vary in length, structure, and location. TRs are short monomeric sequences (up to100 bp) organized directly adjacent to each other, whereas the IRs are dispersed as single copies throughout the genome (97,103). The insertion sequence (IS) IS*6110* is a mobile genetic element that is one of the driving forces for the emergence of Mtb genetic variability. As the number of copies (0-25) and the position of IS*6110* greatly varied on the MTBC genome, it has been a useful and commonly used molecular marker for characterizing MTBC strains (97,104). The IS6110-RFLP analysis showed the highest discriminatory power of all available typing methods to differentiate MTBC strains. Hence, it has been considered the gold standard for distinguishing MTBC strains with more than five copies (105,106). The method is stable, allowing to identify epidemiologically related from unrelated isolates, detecting cross-contamination and mixed infection, and analyzing molecular epidemiology and phylogeny studies (96,97).

The typing method's limitations include being laborious, time-consuming, costly, requiring sub-culturing, requiring many copies, and difficult interlaboratory result comparison (96,107).

Spoligotyping

The original spoligotyping was developed for simultaneous detection and typing of MTBC from clinical specimens to shorten the time taken to culture and type MTBC strains using RFLP (108). Spoligotyping, a PCR-based method, has been widely used to identify and classify the various MTBC genotypes into specific strain families. It detects the presence or absence of 43 unique

spacer-spoligotyping patterns in the direct repeat (DR) region of MTBC by a reverse line blot hybridization approach (96,108).

Spoligotyping accuracy for identifying and genotyping Mtb strains from AFB-positive slides directly from clinical specimens or mycobacterial cultures showed an overall sensitivity and specificity of 97% and 95%, respectively, for the detection of MTBC, and 98% and 96% from clinical specimens alone. The TAT taken for spoligotyping is less by a median of 20 days than the time required for culture-based identification to help manage TB in clinical settings (109). Spoligotyping is mainly used in population-based studies to group phylogeographic MTBC clades. It is also used as a preliminary screening for many Mtb isolates into spoligotype-defined clusters for further typing using high discriminatory power methods like MIRU-VNTR typing (97).

The advantages of spoligotyping over IS6110- based genotyping are the requirement of small amounts of DNA, direct test from clinical samples or MTBC strains shortly after culture into a liquid culture, typing even nonviable cultures, AFB smear slides, or paraffin-embedded tissue sections, and straightforward interpretation of results. Moreover, it is more reproducible than most techniques except for mixed linker PCR and standard IS6110 typing

The low discriminatory power and being unable to identify recent transmissions, failure to identify cross-contamination, and mixed-strain infections when used from clinical samples are the limitations of spoligotyping (96,97,110).

Minisatellite sequences/ Mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) typing

MIRU-VNTR typing overcomes most of the drawbacks of IS6110 RFLP (111). In 2001, a rapid and reproducible 12 loci MIRU-VNTR based typing method was developed but had lower discriminatory power than IS6110-RFLP. In 2006, a 24 loci MIRU-VNTR typing method with a high discriminatory subset of 15 loci was proposed as the new gold standard for molecular epidemiological investigation of TB. It has shown a greater discriminatory power than the original 12 loci MIRU-VNTR and even greater than RFLP when combined with spoligotyping (106,112). A discriminatory subset of 15 loci was found to be 96% of the full 24-locus typing method's total resolution power with equal predictive value for assessing MTBC transmission with that of the IS*6110* RFLP typing technique. Hence the 15-locus typing method is recommended as the new standard for routine epidemiological characterization of MTBC isolates and the 24-locus typing

method as a high-resolution tool for phylogenetic studies (112). A comparison study on the discriminatory power of the 12 and 24 locus MIRU-VNTR typing method showed an increment of the Hunter–Gaston discriminatory index (HGDI) from 0.895 (12 loci) to 0.920 (24 loci) (106).

The RFLP and VNTR typing showed a concordance result of 78.5%, with a slightly higher RFLP discriminatory power level than VNTR typing. However, additional polymorphic guanine-cytosine-rich sequence (PGRS) RFLP typing was used for strains having <5 copies of IS*6110* (111). The 24 loci VNTR typing is automated and can be performed on cell lysate, requiring less DNA and easy to interpret results, which enables easy exchange of data for interlaboratory comparison. It is simpler to efficiently implement high-throughput methods than IS6110-RFLP typing and spoligotyping when using 24 loci (96). Therefore VNTR typing is more suitable than RFLP typing of MTBC in national TB surveillance programs (111). However, MIRU-VNTR typing has some limitations, like the 12 loci MIRU-VNTR based typing has less discriminatory power than IS6110-RFLP typing and is expensive (96).

Whole-genome sequencing using next-generation sequencing (NGS)

WGS using NGS is becoming a commonly used and cost-effective method for detecting DR-TB in developed countries and typing a large number of MTBC strains. It helped conduct more informative epidemiological studies and get genetic information like genomic markers, DR profile, virulence determinants, and genome evolution using a single nucleotide variation (SNVs) in core genomic sequences molecular evolutionary clock (96,113,114).

Though they have limited discriminatory power, several MTBC strain-typing methods have been developed and effectively used in various molecular epidemiological studies during the last two decades. As these typing methods depend on specific polymorphic genetic markers (less than 1% of the genome), they have a low capacity to get full information on recent transmission chains and distinguish genetically closely related strains. Their turnover markers are variable. Hence, these limitations can overcome using NGS/second-generation sequencing (SGS) based WGS technique with the highest-resolution for molecular epidemiological studies (96).

WGS showed an overall sensitivity and specificity of 99.0 and 100.0% for INH, 99.0 and 100.0% for RIF, 94.8 and 65.3% for EMB, 86.2 and 84.4% for PZA, 95.6 and 95.6% for levofloxacin (LFX), 89.5 and 65.3% for moxifloxacin (Mfx), 91.3 and 95.1% for SM, 90.9 and 99.0% for kanamycin (KAM), 90.9 and 100.0% for amikacin (AMK), 88.9 and 98.0% for capreomycin

(CAP), 87.0 and 85.1% for prothionamide (PTO), 85.7 and 99.0% for para-aminosalicylic acid (PAS), and 66.7 and 95.9% for clofazimine (CLO). WGS is a promising approach to foresee resistance to INH, RIF, PZA, LFX, SM, SLIDs, and PTO with satisfactory accuracy. The specificity of WGS in detecting resistance to EMB and high-level resistance to Mfx needed to be amended (113).

WGS typing method has shown several advantages over conventional typing techniques. Among others, WGS has the highest resolution power for large TB outbreaks (115). It is also an effective tool to track TB transmission dynamics and is less time-consuming and cost-effective (96,116). However, the technique has certain shortcomings. The method is still expensive, involves sophisticated bioinformatics systems, requires fast data software processing and large storage capabilities to deliver data to analytics tools and optimization and quality assurance programs (96).

Although WGS showed the highest discriminatory power in molecular epidemiological studies, the other conventional typing methods are still extensively used. In 2015, an all-in-one, web-based bioinformatics tool called Total Genotyping Solution for TB (TGS-TB) was developed that combines both the old and new typing techniques. The TGS-TB facilitates multiple genotyping platforms using NGS for spoligotyping and detecting phylogenies with core genomic SNVs, IS6110 insertion sites, and 43 customized loci VNTR through a user-friendly, simple click interface. TGS-TB provides a more accurate and discriminative strain typing for clinical and epidemiological investigations; NGS strain typing offers a total genotyping solution for MTBC outbreak and surveillance (114).

The automated bioinformatics software used to predict drug resistance from TB WGS data is TBProfiler, MyKrobe, KvarQ and PhyResSE (117). Analysis of WGS data enables comprehensive antibiotic resistance profiling and outbreak surveillance with the highest resolution to identify recent transmission chains. MTBseq, a bioinformatics pipeline for next-generation genome sequence data analysis, enabled to detect variant positions annotated with known association to drug resistance and lineage classification based on phylogenetic SNPs (118).

Drug-resistant tuberculosis

The emergence and dissemination of DR-TB have been striking the global TB control programs. DR-TB is divided into two forms; primary and secondary DR-TB. Primary drug resistance occurs in individuals initially exposed and infected with DR-TB bacteria. Secondary drug resistance is

developed during TB therapy, either because the patient was treated with an inadequate regimen or did not take the prescribed regimen properly or other conditions such as drug malabsorption (119).

Drug resistance in MTBC is caused by an accumulation of spontaneous mutations that interfere with drug-target binding, pro-drug inactivation and target overexpression. However, 30% and 5% of MTBC isolates have DR-conferring mutations outside the known resistance-conferring genes for INH and RIF, respectively (120).

MTBC is a successful pathogen due to the high level of natural DR mechanism of its cell wall, which is impermeable to most chemotherapeutic agents. The factors which are associated with drug resistance are diagnostic delays, treatment supply inconsistencies, unmonitored therapy, nonadherence, incorrect use of drugs, weak infection control program and HIV epidemics (119). These factors act as a selective pressure to facilitate the evolution of resistant MTBC strains, from mono-DR to MDR, XDR, and eventually to totally drug-resistant (TDR) through a sequential accumulation of DR mutations (121). MDR-TB is a TB bacterium resistant to at least INH and rifampicin, the two most potent 1st-line anti-TB drugs. XDR-TB is a form of MDR-TB with additional resistance to any fluoroquinolones (FLQs) and at least one of three 2nd-line injectable drugs (SLIDs) (122). TDR-TB is defined as TB strains resistant to all 1st- and 2nd- line anti-TB drugs tested (123). The mechanism of DR-TB is depicted in **Figure 3**.

There are few new anti-TB drugs. The limited access to these anti-TB drugs or improper combination of an effective regimen of drugs significantly contributes to the growing poor outcome of TB treatment like failure and mortality. Moreover, the DR-TB management is further complicated by the high cost, long duration, and devastating toxicity of currently available 2nd-line drugs (124). Globally, an estimated 20% of TB isolates are resistant to at least one primary anti-TB drug; of them, about 10% are isoniazid-resistant, one of the potent 1st- line anti-TB drugs. Around 5% of TB patients are likely to have MDR-TB, and 10% of them have been categorized as TDR-TB globally (124).

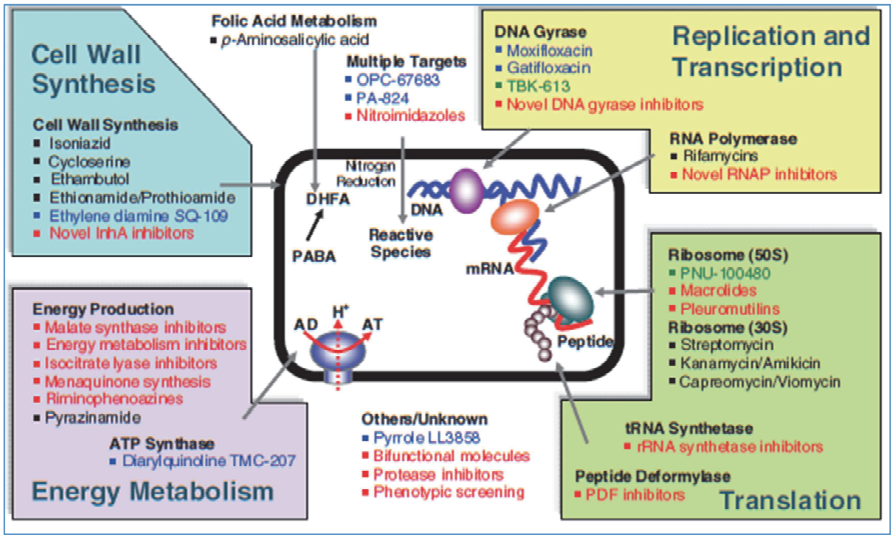

Figure 3: Schematic representation of the mechanism of DR-TB. With permission (125)

Treatment of drug-susceptible TB, MDR-and XDR-TB

The most critical component of TB management is providing standardized treatment to patients (119). TB treatment aims to cure patients, prevent death, limit the illness's complications, prevent relapse and transmission. It is recommended to perform DST at the time of treatment initiation for the anti-TB drugs. However, if baseline DST is impossible, it should be performed on the first positive culture isolated from the patient during treatment monitoring (126).

New TB patients who are presumed or known to have drug-susceptible TB can be treated using standardized daily-based and fixed-dose combination tablets with a 6-month RIF-containing regimen. The two-month intensive phase treatment is with INH, RIF, PZA and EMB (HRZE) and the four-month continuation phase treatment is with HR. According to the recommendation, EMB should be included for all patients with non-cavitary, smear-negative PTB or extrapulmonary TB known to be HIV-negative. However, in the case of tuberculous meningitis, EMB should be replaced by SM. The intensive phase's purpose is to rapidly kill Mtb and make patients less infectious within approximately 10-14 days of initial treatment and symptoms to subside. However, for most patients, the conversion time from sputum smear-positive to smear-negative takes two months. In the continuation phase, these drugs' sterilizing effect eliminates the remaining bacilli and prevents subsequent relapse (127,128).

The recommended treatment regimen for patients with INH-R TB is designated as 6(H) REZ-Lfx, which means a six-month treatment regimen composed of RIF, EMB, PZA, Lfx, and INH can be added if a four-drug fixed-dose combination (HREZ) is used. The 6(H)REZ regimen is prescribed for INH-R TB patients in whom RIF susceptibility is unknown, and LFX cannot be used, or for INH-R TB patients with FLQ-R, LFX intolerance, or other contraindications. The shorter regimen for the treatment of MDR/RR-TB is four-six bedaquiline (Bdq)6-Lfx/Mfx-clofazimine(CLO)-Z-E-Hh-ethionamide(Eto)/5Lfx/Mfx-Cfz-Z-E(shorter all-oral Bdq-containing regimen). However, in the case of FLQ-R, six-nine Bdq- pretomanid (Pa)-linezolid (Lzd) (six-nine month treatment regimen composed of Bdq, Pa, and Lzd-BPaL regimen) is recommended. The other recommended treatment in a specific condition is a more extended regimen for MDR/RR-TB treatment using 18 Bdq(6 m)-Lfx/Mfx-Lzd-Cfz (18-month treatment regimen composed of Bdq for the first six months and Lfx or Mfx, Lzd, Cfz for 18 months) (129).

For MDR/RR-TB patients without previous exposure to 2nd-line drugs for more than one month, with FLQ susceptibility and do not have severe PTB or extrapulmonary TB disease, the ideal treatment option is a shorter all-oral Bdq-containing regimen (4–6 Bdq(6 months)**-**Lfx/Mfx-Cfz-Z-E-Hh-Eto/5Lfx/Mfx-Cfz-Z-E5 month). MDR/RR-TB patients with severe PTB and extrapulmonary TB, additional resistance to FLQ, or exposure to treatment with 2nd-line medicines for more than one month can be treated with an individualized longer regimen designed using the WHO priority grouping of medicines (129).

The six-nine-month treatment regimen composed of BPaL could not be considered a programmatic use worldwide as its efficacy and safety are not well known. However, it may be used under operational research conditions in patients with MDR/RR-TB and additional FLQ resistance who have not had previous exposure to Bdq or Lzd (for <2 weeks). In individual patients for whom the design of an effective regimen based on existing recommendations is impossible, the BPaL regimen may be considered the last option under programmatic conditions (outside operational research). This regimen's application demands high standards for monitoring treatment response and adverse events and providing adequate patient support.

The selection of suitable regimens should be according to the efficacy, safety, patient preference, and clinical judgment and consider DST results of specific drugs, patient treatment history, severity, and site of the disease (129).

Traditionally anti-TB drugs have been classified as 1st- and 2nd-line anti-TB drugs. They are categorized into five different groups based on the efficacy, the experience of use, safety, and drug class according to WHO recommendation, though all drugs in the same group may not be from the same class or may not have the same efficacy or safety **(Table 2)** (119).

Table 2: WHO recommended grouping of anti-TB drugs (119)

GROUP NAME	ANTI-TB AGENT	ABBREVIATION
Group 1. First-line oral agents	Isoniazid	H
	Rifampicin	R
	Ethambutol	E
	Pyrazinamide	Z
	Rifabutin[a]	Rfb
	Rifapentine[a]	Rpt
Group 2. Injectable anti-TB drugs (parental agents)	Streptomycin[b]	Km
	Kanamycin	Am
	Amikacin	Cm
	Capreomycin	Km
Group 3. Fluoroquinolones (FQs)d	Levofloxacin	Lfx
	Moxifloxacin	Mfx
	Gatifloxacin[c]	Gfx
Group 4. Oral bacteriostatic second-line anti-TB drugs	Ethionamide	Eto
	Prothionamide	Pto
	Cycloserine	Cs
	Terizidone[d]	Trd
	Para-aminosalicylic acid	PAS

Group 5. Anti-TB drugs with limited data on efficacy or long term safety in the treatment of DR-TB (This group includes new anti-TB agents)	Bedaquiline	Bdq
	Delamanid	Dlm
	Linezolid	Lzd
	Clofazimine	Cfz
	Amoxicillin/ clavulanate	Amx/Clv
	Imipenem/cilastatin[e]	Ipm/Cln
	Meropenem[e]	Mpm
	High-dose isoniazid	High dose H
	Thioacetazone[f]	T
	Clarithromycin[f]	Clr

Note:

[a] Rifabutin and Rifapentine have similar microbiological activity as rifampicin. Rifabutin is not on the WHO list of essential medicines; however, it has been added here as it is used routinely in patients on protease inhibitors in many settings. Rifapentine is part of a latent TB infection and active TB treatment in some countries but to date is not part of any WHO endorsed treatment regimens.

[b] There has high SM resistance rates in MDR-TB strains; therefore, SM is not considered a 2nd-line anti-TB drugs

[c] Gatifloxacin can have "life-threatening" side effects, including severe diabetes. The drug has been removed from the formula of several countries.

[d] Terizidone has limited program data and effectiveness data as compared to cycloserine.

[e] Clavulanate (Clv) is recommended as an adjunctive agent to imipenem/cilastatin and meropenem.

[f] Limited data on the role of thioacetazone and clarithromycin in MDR-TB treatment has resulted in excluding these drugs as options for Group 5.

Epidemiology of TB

Global burden of TB

TB has continued to be a grave challenge to global public health, being the first killer among infectious diseases (130). TB is a poverty disease; it causes financial distress, vulnerability, marginalization, stigma, and discrimination of the afflicted population, helping the disease to persist throughout history (131,132). The WHO started annual TB reporting in 1997. According to the WHO report, there were an estimated 10.0 million incident cases, 1.2 million TB deaths among HIV-negative people, an additional 208,000 deaths among HIV-positive people, and more

than 95% of these deaths were in developing countries in 2019. Among the new TB cases reported in 2019, 56% were men, 32% were women, 12% were children (aged <15 years), and 87% of the incident cases were from the 30 high TB burden countries. From the total incident cases of 2019, 8.2% were people living with HIV (132). The TB incidence rate reduced by 20% between 2015 and 2020 globally. However, it is not rapid enough to reach the End TB Strategy's first milestone, which targeted a 35% reduction between 2015 and 2020 (132,133).

Epidemiology of drug-resistant TB

DR-TB creates a public health crisis and health security threat that jeopardizes the TB control program in numerous countries, mainly in the high TB burden countries (134). In 2019, there were an estimated 465,000 MDR/RR-TB incident cases, with the estimated global proportion of MDR-TB 78% of the RR-TB. Around 50% of the global MDR/RR-TB incident cases were in India (27%), China (14%), and the Russian Federation (8%). Approximately 182,000 MDR/RR-TB cases died globally in 2019 (132). In the same year, an estimated 13.1% of new cases and 17.4% of previously treated cases had INH-R worldwide. Of the estimated 1.4 million incident cases of INH-R TB in 2019 globally, 1.1 million were susceptible to RIF. Hence, 11% of INH-R incident cases of TB and RIF-susceptible TB can be missed in settings where diagnostic algorithms prioritize RIF-R detection; they do not receive the recommended modified treatment regimen. According to the 105 countries, including 26 among the 40 with a high TB or MDR-TB burden countries report, 20.1% of MDR/RR-TB cases were FLQs-R (132).

TB can affect everyone and anywhere. Factors contributing to increased TB disease include HIV coinfection, malnutrition, overcrowding, alcohol abuse, cigarette smoking, diabetes mellitus, indoor air pollution, and taking immunosuppressive drugs (132,135). Studies indicate that migration to big cities, ethnic minorities, and MDR-TB transmission significantly affects TB disease epidemics (136,137).

The emergence of HIV has created an unprecedented impact on infectious diseases' epidemiology, particularly TB disease. One-third of the HIV-infected persons are estimated to have LTBI. HIV-negative individuals with LTBI have a 10% chance of developing TB disease in their lifetime, while HIV-positive individuals have a 10% chance of developing TB annually (137). TB/HIV coinfection has an impact on the disease progression of both TB and HIV disease. As the viral load increases, TB facilitates the progression of HIV infection to acquired immunodeficiency syndrome

(AIDS) and finally to death. HIV infection, in turn, decreases the CD4 lymphocyte count and accelerates the development of TB disease (138). HIV infection increases TB relapse rate, transmission in the community, mortality, development of extrapulmonary forms of TB and negative sputum smears (138). Thus, HIV infection has also made it difficult to diagnostics of TB (137). This comorbidity must be considered and treated as a global threat of maximum public health priority (**Figure 4**) (137).

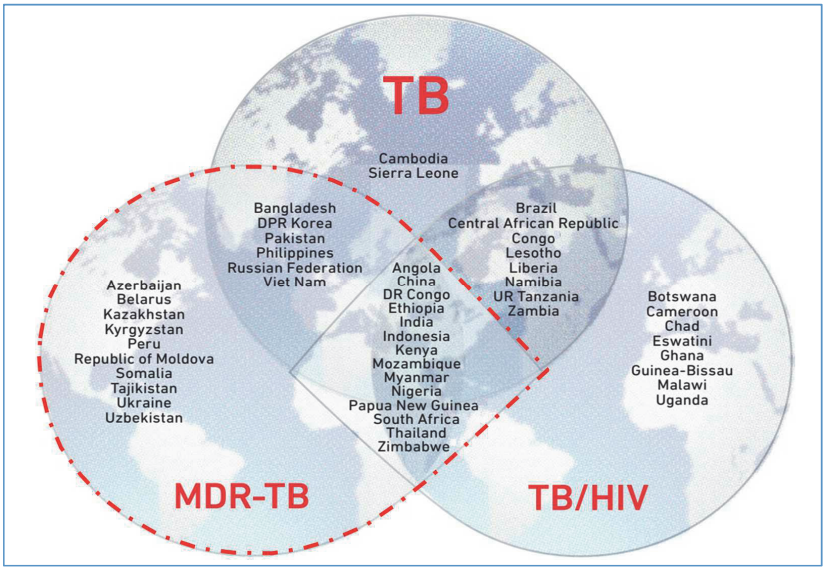

Figure 4: The three high-burden country lists for TB, TB/HIV and MDR-TB defined by WHO for the period 2016–2020, and their areas of overlap. With permission (139).

Global TB control strategies

In 1993, WHO announced TB as a global public health emergency (134). Since 1994, WHO has introduced a cost-effective TB control strategy called Directly Observed Treatment, Short-course (DOTS). This strategy was applicable in the TB control program until 2006 to provide standardized short-course TB treatment to all patients. The principle of DOTS strategy is the patient has to take TB treatment in the presence of DOTS agents, including health workers, the patient's family, and community volunteers, based on the availability of agents and convenience of the patient (140). The five critical components of DOTS are (141):

1. Government commitment to sustained TB control activities

2. Case detection by sputum smear microscopy among symptomatic patients self-reporting to health services.
3. A standardized treatment regimen of six to eight months for at least all confirmed sputum smear-positive cases, with DOT for at least the initial two months.
4. A regular, continuous supply of all essential anti-TB drugs
5. A standardized recording and reporting system allows assessment of treatment results for each patient and the TB control program.

According to a 1996 WHO report, the DOTS strategy was successful, which showed more than 90% of TB patients' cure rate. However, the DOTS strategy did not consider the management of patients with DR-TB (141). In 1999, WHO endorsed an additional strategy called DOTS-Plus to build upon the five components of DOTS strategy and address the management of patients with DR-TB. Following the DOTS strategy's re-definition in 2006, a modified form of the DOTS strategy called the Stop TB Strategy was introduced by the WHO. The Stop TB strategy encompasses six main components (142):

1. Pursue high-quality DOTS expansion and enhancement
2. Address TB/HIV and MDR-TB and other special challenges
3. Contribute to health system strengthening
4. Engage all care providers
5. Empower people with TB and communities
6. Enable and promote researchers

After the end of the Millennium Development Goals period in 2015, the WHO endorsed a new strategy, the End TB Strategy, which spans until 2035: the Paradigm Shift (**Figure 5**) (131).

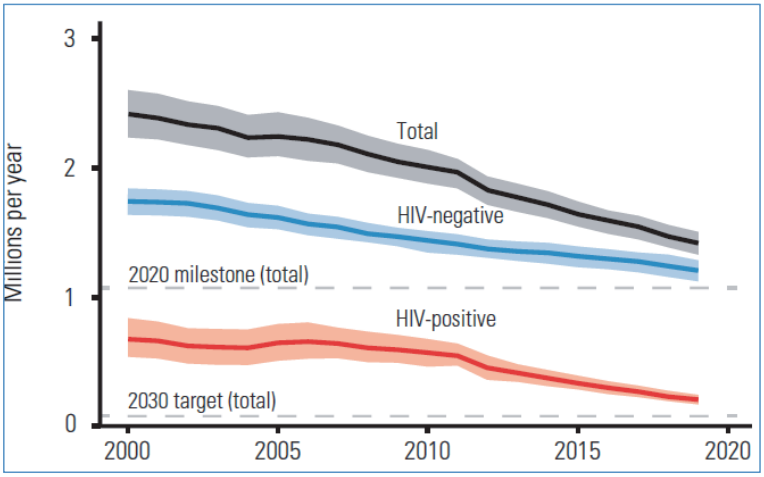

Figure 5: Global trend in the estimated number of TB deaths, 2000–2019. With permission (132) Note: The shaded areas are uncertainty intervals. Horizontal dashed lines mark the 2020 milestone and 2030 target of the End TB Strategy.

Tuberculosis burden in Ethiopia

TB has been identified as one of the significant public health concerns in Ethiopia (143). The country is among the three high TB, TB/HIV, and MDR-TB burden countries. There were 157,000 new TB cases with an incidence rate of 140/100,000 population and 10,000 HIV/TB incident cases reported in 2019. In the same year, the number of MDR/RR-TB cases was 1,400, the number of fatalities from TB was 21,000 for HIV-negative individuals, and an additional 2,800 when including people living with HIV/AIDS (132).

In Ethiopia, MDR-TB is a serious public health problem that needs urgent intervention (144). Treatment interruption and loss of follow-up are the biggest challenges of TB control programs due to their high risk of severe illness, death, disease transmission, poor treatment outcome, and occurrence of drug resistance (145). In Ethiopia, the first MDR-TB patient was admitted for treatment in 2009 in a rehabilitated isolation ward at St. Peter's Hospital in Addis Ababa. In the same year, 45 MDR-TB patients were enrolled in the second phase at St. Peter's hospital. However, there has been a rapid increase of DR-TB in the consecutive years, and 811 MDR-TB patients started treatment in 2012. Of these, nine cases were with XDR-TB (146).

A systematic review and meta-analysis on the prevalence of MDR-TB conducted in Ethiopia reported that 2.2% of newly diagnosed and 21.1% of previously treated patients had MDR-TB with an overall prevalence of 7.24% (0.5 to 46.3%) (147). The various studies conducted in the different parts of the country indicated variability in MDR-TB distribution throughout the country. A study done in the national TB reference laboratory, Ethiopia, showed that 2.3% of new cases and 71.4% of previously treated patients had MDR-TB (148) and Oromia Region had 33.2% MDR-TB cases (13). In Jigjiga (Somali Region), 10.2% of smear-positive were MDR-TB patients (150). In the studies done in Amhara Region, southwest Ethiopia and Addis Ababa, the magnitude of MDR-TB was reported to be 36.3%, 27.7%, and 39.4%, respectively (11,15,16).

Tuberculosis control in Ethiopia

TB centers and sanatoria were established in three urban areas in Ethiopia in the early 1960s. The Central Office (CO) for the National Tuberculosis Control Program (NTCP) was established in 1976. In 1992, the DOTS strategy was started in certain pilot areas in the country (143). Following DOTS initiation, tremendous progress has been brought to the Ethiopian and global TB control program (154).

The health system in Ethiopia is organized in a three-tiered health care delivery system. District health care tier encompassing a primary hospital (n=156), with a catchment population of 60,000-100,000), health centers (n=3335), 1 per 15,000-25,000 population and their satellite health posts (n=15194), 1 per 3,000-5,000 population linked through referral systems, which forms a Primary Health Care Unit (PHCU). The PHCU in the rural settings includes Health posts but not in the urban settings. The second tier contains a general hospital with a catchment population of 1-1.5 million. A specialized hospital with a 3.5-5 million-catchment population forms the third tier of the healthcare delivery system. Private for-profit and non-government organizations supplement the health service coverage and utilization at all levels (143).

According to the Federal Ministry of Health of Ethiopia (FMOH) report, the prevalence of TB in Ethiopia has shown a steady decline since 1995, with an average decline rate of 4% per year. Similarly, TB incidence was estimated to 431/100,000 in 1997 and 1998, with an average decline of 3.9%. TB prevalence for all forms of TB has declined from 426/100,000 in 1990 to 200/100,000 population in 2014 (53% reduction). Likewise, the TB incidence rate has dropped from 369 in 1990 to 192/100,000 in 2015 (48% reduction). The enormous expansion of DOTS centers and the

implementation of the Stop TB Strategy in the national TB program played an imperative role in the declining trend of incidence and prevalence of TB in the country. **Figure 6** shows a trend of higher incidence than prevalence estimates for the last seven years, with a progressive decline in TB mortality and a high treatment success rate, indicating most detected TB cases are incident cases of the same years (143).

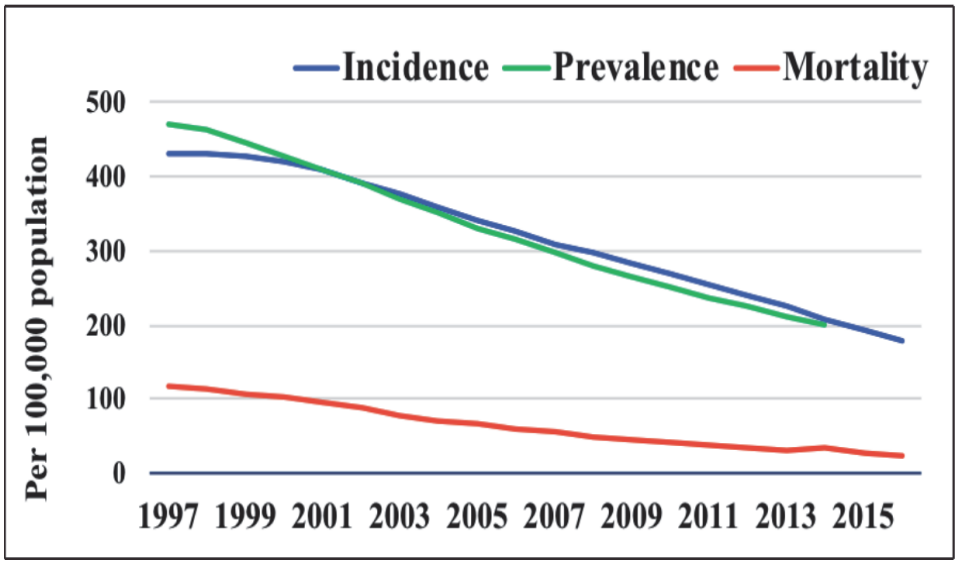

Figure 6: Trends of TB incidence and prevalence rates in Ethiopia: 1990-2015. With permission (143)

The number of notified TB cases has been gradually increasing since 1996, with a peak of 159,017 cases in 2011. However, a downward trend over the last five successive years, as shown in the figure below with 8% (10,000 cases) average annual decline over the last four years (143) (**Figure 7**), and the treatment success rate is depicted in **Figure 8** (143).

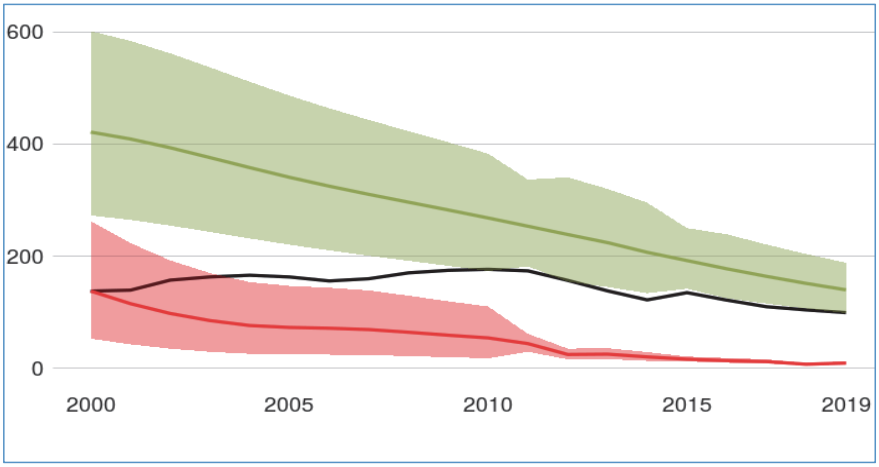

Figure 7: Trend in the annual number of notified TB cases, 2000-2019. With permission (143). **Note** Incidence, New and relapse TB cases notified, HIV-positive TB incidence (Rate per 100 000 population per year)

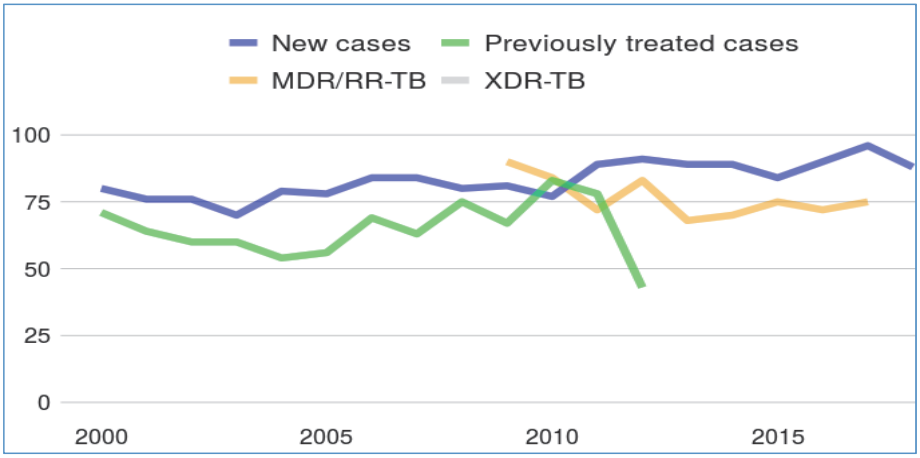

Figure 8: Treatment success rate (%) from 2000-2015. With permission (143)

Although there have been impressive signs of progress to control TB in the country, reports show the presence of tremendous obstacles to tackle TB. The barriers for case finding include inadequate resources such as shortage of healthcare providers, inadequate diagnostic equipment and supplies, and limited access to TB diagnostic services like the absence of nearby TB diagnostic health facilities and diagnostic delays in the health system. Moreover, this research finding identified 11,

13, and seven health facilities without trained laboratory professionals, clean water supply, and electricity, respectively. Additionally, the topography difficulty, the absence of proper roads, inadequate collaboration with other education sectors, a turnover of laboratory professionals, and low community mobilization were identified as the origin of some of these barriers (143). In Ethiopia, studies revealed that a considerable number of undiagnosed TB patients with an estimated point of prevalence 79.7/100,000, and the delayed case detection and treatment of MDR-TB cases might, in turn, contribute to the transmission of the disease in the community (143,155).

TB burden and control strategies in Tigray

Tigray Region is one of the nine Regional States in Ethiopia, located in the northern part of the country. TB is the leading cause of morbidity and mortality in Tigray. In one study, the point prevalence of bacteriologically confirmed TB was estimated to be 216 per 100,000 population in the region (156). A retrospective study on culture-positive presumptive MDR-TB samples sent to the Tigray Health Research Laboratory (THRL) indicated that the MDR-TB proportion was 54.6% (157). Another cross-sectional study on presumptive MDR-TB revealed a high proportion (18.5%) of MDR-TB in the Region (158).

The health system organization and NTCP of Ethiopia are decentralized to all regional states (143). Though in Ethiopia, the treatment success rate was high in the previous years, there were variabilities across the country's regions that impel tailored interventions. According to the routine TB control program data, treatment failure and Tigray's death rate was above the national average in the previous years. Hence, the need for sub-national targeted support of the population and geographic settings ensures equitable outcomes (143).

Study findings conducted in prisons of the Tigray Region revealed that at least half of the prisoners with symptomatic pulmonary TB were undiagnosed (159). Regardless of the role of health extension workers to halt TB dissemination in the community, findings showed that a low (21.7%) proportion of household contacts were screened by the health extension workers (160). Hence, there should be re-enforcement of the TB prevention programs and strengthening the DOTS strategy and laboratories for DST in the region to prevent the emergence and transmission of MDR-TB in the community (157).

Knowledge gaps

Several review papers on the burden of MDR-TB in Ethiopia revealed a considerable variation in the burden and associated factors of MDR-TB across the country (144,161,162). A review done in Ethiopia found diversified strains and lineages of Mtb, and the frequency of occurrence of these strains and lineages were variable in different regions of the country (16). Moreover, a molecular analysis of Mtb strains at St Peter's TB Specialized Hospital in Addis Ababa, a National Referral Hospital, and a similar study in Northwest Ethiopia showed high Mtb diversity (163,164). As Ethiopia is a demographically complex country, it is unlikely that the strain composition found in one part of the country would be the same as in the other regions (17). The variability in DR distribution in the different regions of the country was linked to the association between DR and Mtb lineage diversities (165), and the association of the various Mtb lineages with geographic locations and sociodemographic background of study participants (166). Hence, further studies in the respective regions are needed to get adequate data to strengthen laboratory diagnosis of TB, intensified case finding, and prompt treatment of DR-TB patients (163).

However, there are limited studies on the molecular epidemiology of multidrug-resistant TB among PTB patients in the country (17,163–166) and there is no study in the study area so far. Therefore, there is a need to investigate the molecular epidemiology of MDR-TB among PTB patients in the Tigray Region, Ethiopia.

Aims and objectives

The study aimed at describing the genetic diversity and molecular epidemiology of multidrug-resistant MTBC among pulmonary TB patients and link these data to demographic and health information from TB patients in the Tigray Region, Ethiopia.

This aim was translated into research papers with the following specific objectives:

1. To describe the demographic and clinical characteristic of pulmonary MDR-TB patients in Tigray Region, Ethiopia (Paper-I)
2. To describe the frequency and patterns of first- and second-line drug resistance-conferring mutations of MTBC among pulmonary TB patients in Tigray Region, Ethiopia (Paper-II)
3. To identify the diversity and distribution of MTBC genotypes in Tigray Region, Ethiopia (Paper-III).

The conceptual framework for the study

The conceptual framework that shows the host and bacterial factors associated with drug resistance are schematically presented in **Figure 9.** Factors associated with drug resistance are socio-demographic, behavioral characteristics, co-morbidities, spontaneous mutations, the genetic background of MTBC strains, and other external factors. The external factors include doctor-related, patient-related and health system-related factors. These factors, directly and indirectly, contribute to the evolution of DR-TB.

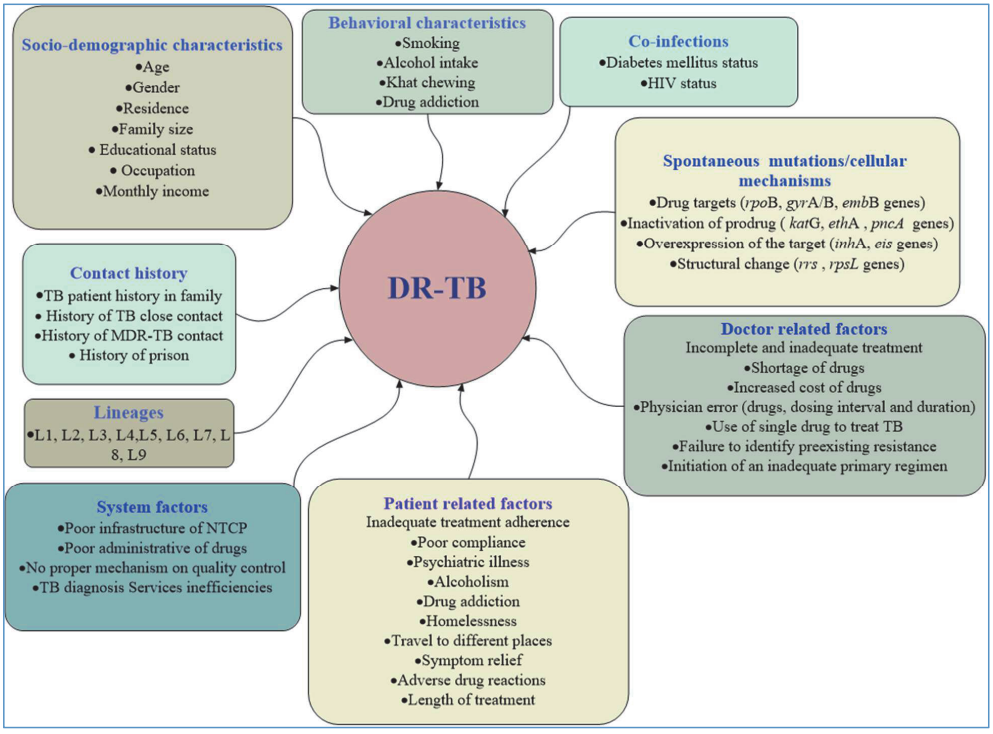

Figure 9: Conceptual framework of the study

Materials and methods

Study area and setting

Ethiopia is one of the East African countries, situated at a range of 3º to 15º N and 33º to 48º E with a total area coverage of about 1.12 million km^2 and an estimated population of 100.8 million people (167). In Ethiopia, the predominant livelihood is based on agricultural activities, and the rural dwellers account for 80% of the population. The country is administratively divided into 10 Regional States and two City Administrative Councils (Addis Ababa and Dire Dawa), and Addis Ababa is the country's capital city. The Regions are subdivided into Zones, Woredas, and *Kebeles* (the lowest administrative unit).

The study was conducted in the Tigray Regional State. The region is located at latitude 14.2 and longitude of 38.8 degrees, 12º15 to 14º57 N, and 36º27 to 39º59 E at the northern highlands of Ethiopia, and the total area coverage is 54,573 km^2. The capital city of Tigray is Mekelle, which is 783 km away from Addis Ababa. Tigray has an estimated total population of 5.5 million (167). About 80% of the population lives in rural areas where the people's livelihood mainly depends on agriculture. There are seven zones (one special zone, Mekelle), 52 districts, and 814 *Kebeles*.

The region's health infrastructure includes 40 hospitals, 223 health centers, and 710 health posts serving Tigray and neighboring regions. All the hospitals and health centers are equipped with TB diagnostic facilities with AFB smear microscopy, and some hospitals have additional GeneXpert TB diagnostic facilities. The Tigray Health Research Institute (THRI) is the only facility providing TB culture and drug susceptibility testing in the Region. The Stop TB with the DOTS strategy is implemented to control TB in the Region. At the time of this study, there were 51 specialist doctors, 87 general practitioners, 3092 nurses, 792 midwives, 562 laboratory technologists, more than 1539 health extension workers, and other professionals providing preventive and curative services in the institutions mentioned above (168). The DOTS-Plus program has been introduced in all health facilities. Currently, there are nine treatment initiation centers and 62 treatment follow-up centers for MDR-TB in the Region.

The study was conducted in six hospitals: the Alamata Hospital, Southern Zone; the Mekelle Hospital, Mekelle Special Zone; the Adigrat Hospital, Eastern Zone; the Adwa Hospital, Central Zone; the Shire/Suhul Hospital, Northwestern Zone and the Humera/Kahsay Abera Hospital, Western Zone) of the Region **(Figure 10)**.

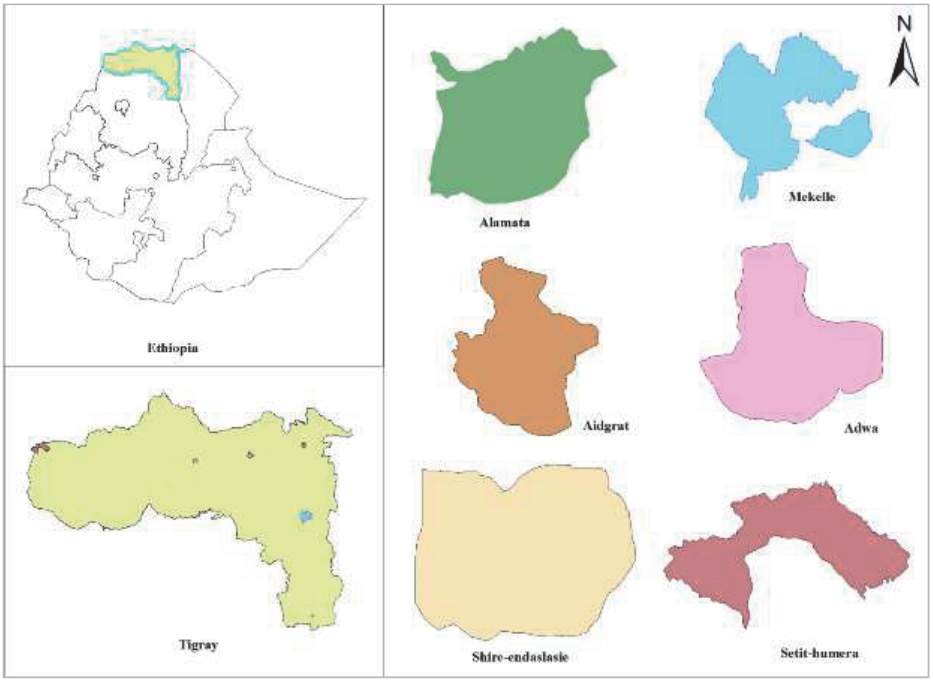

Figure 10: Map of the study area showing locations of hospitals

Study design, population and inclusion criteria

The study was a hospital-based, cross-sectional study conducted from July 2018 to August 2019. The source population was all presumptive PTB cases in the Tigray Region during the study period. The study population was all presumptive PTB cases who visited the selected health facilities in the region during the study period. PTB patients' who were not under treatment during the study, ≥15 years of age, who were GeneXpert-positive and could provide written informed consent, were included in the study. Critically ill patients from whom sociodemographic, clinical data, and sputum samples could not be obtained, patients <15 years of age and extra-pulmonary TB cases were excluded from the study.

Sample size determination

The sample size was determined through the required minimum number of MDR-TB patients enrolled in the study. It was estimated to be between 30-40 cases to establish a sample with enough MDR-TB isolates to allow sufficient power to find different varieties of MDR-TB isolates for

molecular analysis and find a genetic diversity of MTBC. Based on an expected level of 10% (162) MDR-TB among TB patients, we aimed to recruit at least 300 TB patients for the study. This allowed us to sample enough patients for the molecular studies and maintain a reasonable precision level for the prevalence estimates. Without adjusting for clustering, the estimate's precision would be at 6.7%-14.2% (95% CI, a relative precision of ± 3.3%).

Six government hospitals from the Tigray Region were selected based on the GeneXpert diagnostic technique's availability as the primary test for presumptive PTB patients from the main zones of the region. The selected hospitals were the only hospitals with the GeneXpert test to diagnose all presumptive PTB patients at the start of the study. These hospitals were also giving diagnostic services for other neighboring health institutes that do not have a GeneXpert test. The standard practice of the hospitals was the first testing of all presumptive PTB patients by GeneXpert test. If the GeneXpert test result showed no resistance, the patient immediately starts on 1st-line-TB treatment. If the result were RIF-R TB, the patient linked to the MDR-TB clinic, where he/she would be asked to provide a sputum sample for mycobacterial culture before initiating MDR-TB treatment.

For this study, sputum samples were collected for culture and DST from all consecutive GeneXpert positive PTB patients who fulfilled the inclusion criteria. A consecutive sampling technique was employed to recruit the study sample in all the hospitals until the required sample size was obtained.

Data collection

Socio-demographic, clinical data, history of previous medical illnesses, and behavioral factors were collected from patients using a pre-tested structured questionnaire. Adequate training in data collection was provided to data collectors before the commencement of the study.

GeneXpert® MTB/RIF assay

From each patient, a 4 ml sputum sample was collected "on-the-spot" and treated with a sample reagent (SR) containing NaOH and isopropanol according to the recommendation of FMOH (32). The treated samples were transferred into the cartridge, and the cartridge was loaded into the GeneXpert instrument following the manufacturer's instructions (169). The process identifies Mtb DNA and RIF-R inducing mutations in the RNA polymerase beta (*rpo*B) gene in the Mtb genome in a real-time format using fluorescent probes called molecular beacons.

Sputum collection for culture and culture identification

From every GeneXpert MTB/RIF assay positive participant, 5-10 ml sputum sample was collected according to the recommendation of FMOH (170). The collected sputa were packed correctly and kept at 4°C for transportation in an ice bag to THRI according to WHO recommendation for transporting biological substance; category B, UN-3373. The processed sputum samples were cultured on LJ egg medium and liquid culture *Mycobacterium* Growth Indicator Tube 960 culture following the standard operational procedures. Identification of mycobacteria were performed by colony morphology, AFB staining, Capilia antigen test, and inoculation on blood agar plate.

Drug susceptibility testing for 1st- and 2nd- line anti-TB drugs using LPA

Drug-susceptibility testing for 1st- and 2nd-line anti-TB drugs were done using LPA genotypic method following the manufacturer's instruction (GenoType® MTBC; Hain Life science, Nehren, Germany) (171). DNA extraction from culture using GenoLyse® kit (A and B) and amplification Mixes A (10 µl) and B (35 µl) preparation was performed in a DNA contaminant-free working areas. H37Rv as a positive quality control strain and distilled water as negative quality control was used. After amplification, the amplicon was detected with various procedures by adding different reagents to the strip. The strips formed color bands after adding the final substrate reagent (171).

DNA extraction and whole-genome sequencing

After completing the work in Tigray, samples were brought to the University of Oslo to sub-culture and extract DNA and preparation for WGS. DNA was extracted from 74 MTBC culture-positive isolates (26 MDR-TB and 48 mono/susceptible isolates by LPA) for WGS. The susceptible isolates were selected based on stated criteria.

DNA was extracted using a combination of physical and chemical methods (172). Quantitative and qualitative measurements of the DNA were performed using Qubit® 3.0 Fluorometer and NanoDrop spectrophotometers (Thermo Fisher Scientific) (173), respectively. The DNA library was prepared using Kapa DNA Library preparation kit and sequenced by Illumina-NextSeq 500, with a Paired-End read length of 2 x 75 (Genohub Inc.USA). The patient recruitment procedures of the studies linked to the thesis are briefly described in **Figure 11.**

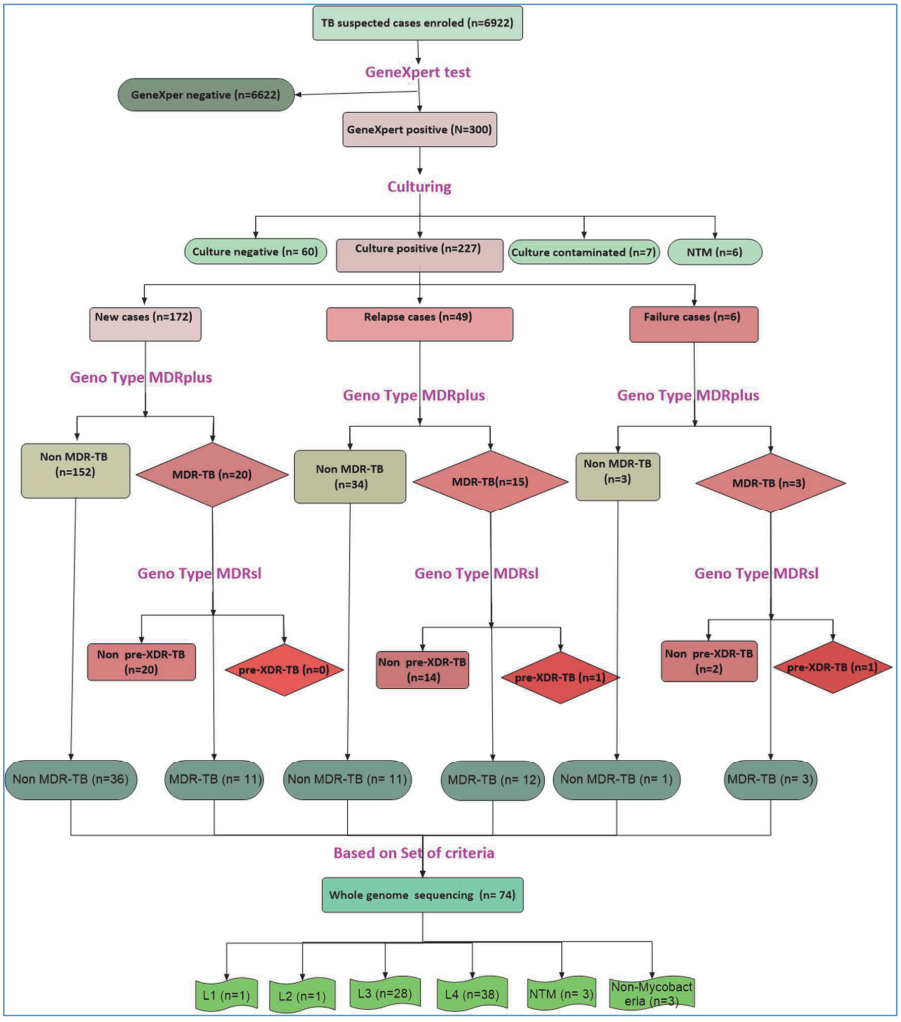

Figure 11: The overall flow of the study participants' recruitment and selection process of the studies reported in the thesis

Data entry and statistical analysis

The complete and cleaned data were entered using the Epidata 3.1 data entry software. After cleaning and validation, data were transferred into Stata (SE 16 for Windows) for further statistical analysis. Frequencies and proportions were presented in tables. Univariable and multivariable logistic regressions were used to assess the associated factors of MDR-TB. Sequence reads were

aligned to reference genome H37Rv (103) using the snippy pipeline (v4.3.6) (174). Individual isolates were typed using the Coll *et al*. scheme (175) with the program colltyper (v0.7) (176). Microbe predictor (v0.7.0) was used to screen for mutations involved in antimicrobial resistance (AMR) (177) and independently verified with TB-profiler (178). Snippy-core (v4.3.6) was used to get whole-genome alignments of all isolates. From this, known repeat regions such as PE/PPE regions were masked, and the resultant reduced alignment was used to create a phylogenetic tree in FastTree (v2.1.10) (179) using the GTR evolutionary model. Additionally, variant call format (VCF) files from Snippy was used to generate a visualization in Nextstrain (180). This allowed in-depth inspection of AMR emergence on branches of the phylogenetic tree. Finally, we used auspice.us to visualize these results.

Quality assurance and quality control

All laboratory analysis was carried out following standard operating procedures. Both the solid culture and LPA procedures were checked and validated. The quality of the WGS data was assessed with FastQC (v0.11.8) (181) and aggregated with MultiQC (v0.4) (182). No samples were found to have adapter contamination above the 0.1% level. All isolates were verified as MTBC by MASH (v2.1) (183) and verified by kraken (v2.0.7-beta) using the minikraken database (RefSeq bacteria, archaea, viruses build 2019-04) (184).

Ethical considerations

Ethical clearance was obtained from Mekelle University, Ministry of Science and Higher Education, Ethiopia, and the Regional Committee for Medical Research Ethics in Eastern Norway. Written informed consent was secured from all study participants before the commencement of the study.

Results

Characteristics of Pulmonary Multidrug-Resistant Tuberculosis Patients in Tigray Region, Ethiopia (Paper-I)

From the total 300 sputum samples collected, 227 (75.67%) were MTBC culture positive. The study participants' median age was 30 years, ranging from 15-85 years. The majority of the participants include 196 (65.3%) males, 220 (73.3%) new TB cases, 112 (37.3%) in the age group of 25-34 years, 140 (46.7%) married, 167 (55.7%) urban dwellers, 98 (32.7%) had an elementary school, 84 (28.0%) self-employed and 95 (31.7%) with no monthly income. LPA analysis on DR pattern of 1st- and 2nd- line anti-TB drugs indicated that the overall MDR-TB was high (16.7%), with a higher proportion of MDR-TB among previously treated cases (32.7%) compared to the new cases (11.6%). In this study, the proportion of pre-XDR-TB (5.3%) was alarming. Of the total 227 TB positive patients, the proportion of coinfection with TB/HIV, non-MDR-TB/HIV, and MDR-TB/HIV was 33%, 17.5%, and 21.1%, respectively. The current study showed that the zonal distribution of MDR-TB differed, highest in the Southern Zone (21.1%), followed by the Mekelle Special Zone (18.4%). Multivariable logistic regression analysis showed that a history of previous TB treatment, cigarette smoking habit, and intermittent fever was associated with MDR-TB development.

Frequency and Patterns of First- and Second-Line Drug Resistance-Conferring Mutations of Mycobacterium Tuberculosis Isolated from Pulmonary Tuberculosis Patients in a Cross-Sectional Study Tigray Region, Ethiopia (Paper-II)

Drug resistance patterns based on the type of TB cases revealed that the predominant RIF-R Mtb were isolated from new (52.5%) followed by relapse (40%) and treatment failure cases (7.5%). A similar pattern was observed for INH-R Mtb, where the majority were detected from new (56%), then relapse (36.6%), and failure cases (7.3%). Furthermore, the highest proportion of MDR-TB was from new cases (52.6%), followed by relapse cases (39.5%) and failure cases (7.9%). An equal proportion of pre-XDR TB (5.3%) was observed among relapse and treatment failure cases.

Resistance-conferring mutations were observed in 40 (17.6%), 41 (18.0%), and two (5.3%) isolates for RIF, INH, and FLQs, respectively. Mutations resulting in an amino acid change within the 81-bp core region of the RNA polymerase β-subunit (*rpo*B) gene were found in 92.5% of RIF-R Mtb

strains, which include codons S531L (70%), H526Y (10%), H526D (7.5%) and D516V (5%). Unknown mutations were detected in 20% of RIF-R isolates characterized by missing wild-type probes without the development of corresponding mutant bands. The *kat*G gene codon S315T (78%) and *inh*A gene codon C15T (5%) were responsible for the INH-R isolates, and 19.5% of INH-R isolates showed unknown mutations. Both the pre-XDR isolates showed mutations at *gyr*A codon D94Y/N, and one of the isolates had additional mutations at *gyr*A codon D94G and the other one additional mutation at *gyr*A codon A90V. No Mtb isolate had mutations at both *kat*G and *inh*A promoter regions simultaneously, and none of the isolates showed DR associated with mutations at the *gyr*B promoter region.

Whole-Genome Sequencing Revealed Disputed *rpo*B mutations and Lineage-Specific Association with Drug-Resistance of *Mycobacterium tuberculosis* in Tigray Region, Ethiopia (Paper-III)

Out of the 74 isolates subjected to WGS, 68 isolates were Mtb, three NTM (two *M. simae* and one *M. elephantis*) and three non-mycobacteria. The 68 Mtb isolates were used for further analysis in this paper. The median age of the 68 participants was 30 years, ranging from 16-66 years. Of these, 47 (69.1%) were males and 21 (30.9%) females.

WGS uncovered the lineage makeup and distribution in the Tigray Region. L4 (38; 55.8%) was the dominant lineage, followed by L3 (28, 41.2%), and an equal proportion of L1 (1; 1.5%) and L2 (1; 1.5%). The most frequent sub-lineage observed was CAS (26; 38.2%) followed by Ural (20; 29.4%), Haarlem (8; 11.8%), LAM (3; 4.4%), Uganda (2; 2.9%), CAS1-Kili (2; 2.9%), X-type (2; 2.9%), Cameroon (1; 1.5%), T3 (1; 1.5%), EAI2 (1; 1.5%), Beijing (1; 1.5%), TUR (1; 1.5%) and LAM7-TUR (1; 1.5%). The transmission index (RTI) and proportion of clustering were 80.9% and 91.2%, respectively. L4 and particularly sub-lineage Ural were associated with any DR to 1st- and 2nd- line anti-TB drugs (P<0.05).

Of the 25 isolates resistant to RIF, the predominant mutations occurred at *rpoB* codon S450L (531) (18; 72%) followed by H445N (526) (2; 8%). Mutations at *rpoB* codons H445Y (526), H445D (526), D435Y, D435V (516) and L430P (511) occurred at one (1; 4%) isolate for each. Except for one isolate which showed mutation at *fab*G1gene codon C-15T (1; 3.8%), all the INH-R isolates showed mutation at *katG* gene codon S315T (25; 96.2%).

The main resistance-conferring mutations for the 18 isolates resistant to EMB were found at *embB* gene codon M306I (4; 22%), D328G/Y (4; 22.2%), G406A (3; 16.7%), M306V (2; 11%), Q497K (1; 5.6%) and D354A (1; 5.6%). Double mutations were detected at *embB* gene codons M306I and G406A (1; 5.6%), G406A and D1024N (1; 5.6%), and M306I and G406S (1; 5.6%). The dominant mutation for SM-R isolates (24 isolates) was detected at *rps*L gene codon K43R (14; 58%). Three (12.5%) isolates showed double mutation at both *rpsL* gene codon K88T and *gid* gene codon G69D. Additionally, SM-R conferring-mutations were observed at *rps*L gene codon K88T, *rrs* gene codons A514C and C517T, *gid* gene codons 115del, 351del, 102del and G69D from one (4.2%) isolate each.

Resistance-conferring mutations to pyrazinamide (PZA) resistant isolates (10 isolates) occurred at *pncA* gene codons V139A (2; 20%) and F58L, F13L, G46G, P69L, T76P, V180F, Q141P and D49A in one isolate each (10%). One isolate (10%) showed silent mutation at *pncA* gene codon G46G. Among the 11 isolates resistant to Ethionamide (ETH), five (45.5%) isolates showed resistance-conferring mutations at *ethA* gene codon M1R, one (9%) at *fab*G1 gene codon C-15T and five (45.5%) showed mutations at other codons (1407del and 1341del).

The drug resistance-conferring mutation for 2nd-line anti-TB drugs occurred at *gyr*A codon D94G for FLQs resistance and *rrs* gene codon A1401G for injectable 2nd- line anti-TB drugs (AMK, KAM and CAP) in one isolate. Additionally, mutation at *tly*A gene codon N236K was detected in another isolate resistant to CAP.

Discussion

The current study is the first study that addresses the genetic diversity of MTBC strains, the magnitude, zonal distribution, and associated factors of MDRT-TB in the Tigray Region of Ethiopia. Knowing the type of MTBC strains circulating in the region and the status and linkage of the specific lineages with drug resistance is crucial to understand the challenges and contribute to the improvement of the TB control program in the study region.

The presented study indicated that the presence of a high proportion of overall MDR-TB (16.7%) with a higher proportion of MDR-TB among previously treated cases (32.7%) than new cases (11.6%) in the Tigray Region. The overall proportion of MDR-TB among new and previously treated cases in this study is much higher than the national (147) and global MDR/RR-TB reports (132). The prioritized approach to developing feasible and cost-effective strategies to prevent and control MDR-TB is to identify the underlying reasons for MDR-TB emergence (185). DR-TB evolved due to diagnostic delay, TB treatment supply inconsistencies, nonadherence, incorrect use of anti-TB agents, inefficient infection control, and the HIV epidemic (119). The factors associated with the MDR-TB development in the present study were history of previous TB treatment, cigarette smoking habit, and intermittent fever. The DOTS-Plus strategy has an incredible value to prevent the emergence of drug resistance and health extension workers (HEWs) play a key role in implementing the DOTS-Plus strategy (140).

However, the low efficiency of HEWs in Tigray Regional state (186) could affect service access to TB patients, affecting early case detection and treatment success rate (65, 66). Another indicator of the ineffectiveness of the role of HEWs is the screening of a low proportion of household contacts of TB (160) and the presence of a large number of undiagnosed TB patients (159) in the Tigray Region. These factors and the high proportion of TB/HIV coinfection in the current study could presumably contribute to the emergence and dissemination of DR-TB in the community. Hence, according to our finding, the highest proportion of MDR-TB was from new cases (52.6%), followed by relapse cases (39.5%) and failure cases (7.9%).

Knowledge of the possible mutations that confer DR at specific drug targets is a key strategy for rapid detection of drug-resistance and containing the disease dissemination. The acquired DR mechanism of MTBC is associated with the accumulation of spontaneous mutations at the *rpo*B gene, *kat*G/*inh*A gene, and *gyr*A/B genes for RIF, INH, and FLQs, respectively. Similarly,

according to the LPA analysis, 92.5% of RIF-R Mtb strains showed mutations at the 81-bp core region of the *rpo*B gene with the frequent mutation at codon S531L (70%) followed by codons H526Y (10%), H526D (7.5%) and D516V (5%). Similarly, previous studies reported the dominance of mutation at S531L (450), followed by H526Y/D (445) and D516V (435) worldwide (189–191). Mutations that occurred at codons 516, 526, or 531 are associated with high-level RIF-R, while mutations at codons 511, 518, 522, 533 confers low-level RIF-R (192). RIF-R's rapid detection is of particular importance since it also represents a valuable surrogate marker for MDR-TB, which is a tremendous obstacle to TB therapy. A study showed that 90.2% of RIF-R strains examined were also resistant to INH, suggesting that RIF-R is a good predictor of MDR-TB (193).

Our study revealed that *kat*G gene codon S315T (78%) and *inh*A gene codon C15T (5%) were responsible for the INH-R isolates. On the contrary, a study showed that *kat*G gene mutations were responsible for 91.2%, *inh*A gene mutations for 7%, and *ahp*C gene mutations for 1.8% resistance to INH (194). Overall, if the mutation occurred at the *inh*A gene, INH could still be effective at a high dose; however, if the mutation is present at both *inh*A and *kat*G genes, INH is no longer useful (195).

Unknown mutations were detected in 20% of RIF-R isolates and 19.5% of INH-R isolates. This finding is supported by a prior report indicating about 30% and 5% of Mtb isolates having DR conferring-mutations outside the known resistance-conferring genes for INH and RIF, respectively, out of the scope of LPA (120).

Resistance to FLQs is due to point mutations in the *gyr*A and *gyr*B genes, encoding the DNA gyrase's two subunits (A and B). The main resistance-conferring mutations to FLQs occur in a short segment termed the Quinolone Resistance Determining Region (QRDR) in the *gyr*A gene codons 85 to 96 and less frequently *gyr*B gene codons 500 to 538 associated with a low level of FLQs-R (196,197). Likewise, in the present study, both the pre-XDR isolates showed mutations at *gyr*A codon D94Y/N, and one of the isolates had additional mutations at *gyr*A codon D94G and the other one additional mutation at *gyr*A codon A90V. No mutation was found in *gyr*B in our study, similar to a study from Morocco (198).

According to WGS analysis, the overall proportion of L4 was the highest, followed by L3 and an equal proportion of L1 and L2. However, there were Zonal variations in composition and proportion of Mtb strains in the Tigray Region. The dominance of L4, followed by L3, is in

agreement with nationwide reviews in Ethiopia, which showed that in all regions, L4 was the most frequently isolated (16,199). L1 is found along the Indian Ocean, L2 is found mainly in East Asia. L3 is common in Central Asia and prevalent in East Africa, and L4 is found mainly in Africa, Europe and America (12). The three frequently isolated sub-lineages were CAS (38.2%) Ural (29.4%), Haarlem (11.8%). In contrast, according to the nationwide review reports, the most dominant sub-lineage in all Ethiopia regions were reported to be T, followed by CAS and Haarlem, except in Benishangul-Gumz Region (16,199). The CAS sub-lineage is dominant in the Indian subcontinent, South-East Asia, the Middle-East and East-Africa (200). In the present study, specialist Mtb strains like Uganda and Cameroon, which occurred in specific countries, were isolated, suggesting the probability of these strains disseminating to other countries.

The various geographic distribution of generalist and specialist sub-lineages could be influenced by intrinsic biological factors, extrinsic factors such as human migration, or both (201). Phylogeography encodes the spatial and temporal distribution of population structure based on the ecological and biological requirements which can decipher evolutionary processes. The spatial genetic variability depends on population structure mechanisms like population contraction, population expansion, gene movements, climate fluctuations and the physical landscape. The ecological heterogeneity helps the organism colonize new sites and induce adaptive genetic changes with a corresponding mutation at specific loci (202). Though some genotypes already existed in the African continent before the European contact, a number of genotypes were introduced to Ethiopia through human migration and trade (12).

The RTI and proportion of clustering were very high, accounted for 80.9% and 91.2%. L4 and particularly sub-lineage Ural were associated with any DR to 1st- and 2nd-line anti-TB drugs ($P<0.05$), suggesting high ongoing DR-TB transmission in the community. The high RTI and dominance of L4 supported the high proportion of MDR-TB among new PTB cases in the present study (203).

The association of DR with the Mtb strains varied greatly. A study reports shown the association of Haarlem (163), Beijing (166,204), and LAM (205) with DR. The different DR-Mtb strains were associated with different geographic areas (166). Factors associated with drug resistance are poor public health infrastructure, poor TB control program management, inadequate treatment, inefficient infection control, and the HIV epidemic (119). These factors act as a selective pressure

to facilitate the evolution of resistant Mtb strains through a sequential accumulation of DR mutations (121), and the associated factors varied by geographic regions (206).

Diagnosis of DR-TB remains a challenge to the TB control programs. Phenotypic DST is slow and expensive, and genotypic assays detect only certain drugs' common resistance-conferring mutations. However, WGS can detect all common and rare mutations conferring-resistance to all 1st- and 2nd- line anti-TB drugs (207). Though the proportion varied, the common mutations to RIF, INH, and FLQs detected by LPA were similar to the WGS findings. However, WGS revealed a significant proportion of disputed mutations (16%) that cause RIF-R at *rpoB* gene codons H445N (526), D435Y (516) and L430P (511), which were not detected using LPA analysis. The occurrence of disputed *rpoB mutations is* clinically and epidemiologically highly relevant (208). According to the WGS analysis, 96.2% of mutations that conferred INH-R occurred at *katG* codon S315T, which differed from LPA results that showed 78% of the mutation occurred at *katG* codon S315T and 19.5% unknown mutations. Mutations like *tlyA* gene codon N236K that confers CAP-R (an isolate susceptible to all other drugs) were detected by WGS, which LPA cannot detect. Similarly, WGS analysis enables detecting mutations that ensue resistance to ETB, SM, PZA and ETH with the predominant mutations at *embB* M306I/V, *rps*L K43R, *pncA* V139A, *ethA* M1R, respectively. Both the pre-XDR isolates showed mutations at FLQs by LPA. However, according to WGS analysis, one isolate showed mutation at *gyr*A D94G that confer-resistance to FLQs. Another one showed mutation at *rrs* A1401G that cause resistance to injectable 2nd-line anti-TB drugs. These findings enlight the clinical usefulness of WGS for the rapid diagnosis and detection of mutations that confer resistance to 1st- and 2nd-line anti-TB drugs.

Methodological considerations

Diagnostic methods used

The diagnostic methods used have different capacities and may be used in different settings. As we have used them in different parts of the study, results may lead to different interpretations. All techniques used have been approved and recommended by international health organizations, as WHO and others.

The Genexpert method was used as a screening technique to collect sputum samples from PTB patients for culturing and DST. Genexpert's strength is that it is a sensitive technique to identify MTBC and detect RIF resistance (a surrogate marker for MDR-TB) simultaneously within a short

time (≈ 2 hours) than AFB; these techniques are available in all the study hospitals. However, Genexpert cannot detect INH resistance.

Following growth and identification, MTBC was subjected to DST using LPA for 1st- and 2nd- line anti-TB drugs. The strength of LPA for DST is providing results in a short period and currently the recommended technique by WHO and the national TB control program for detection of DR comparing with culture. The LPA's disadvantage is that it only detects RIF and INH resistance from the 1st-line anti TB drugs and only detects mutations at the hot spot regions that confer DR. In contrast, several unknown mutations can be discoverable by WGS.

The extracted DNA from the selected strains were subjected to WGS for lineage identification and detailed identification of mutations that confer drug-resistance. The WGS has higher discriminatory power for strain characterization than the other typing methods, detects mutations conferring drug-resistance throughout the isolates' genome, detects drug-resistance for all anti-TB drugs, and is fast. However, its limitation is the high cost; thus, we could not perform WGS for all isolates. Given a sufficient quality of DNA extraction, WGS is an automated and reproducible technique of high reliability.

Internal validity

Data was collected by recruiting patients in a cross-sectional study employing among the study participants from six hospitals representing the Tigray region. The main objective was to identify the variability and find enough genetic patterns among the isolates studied. The prevalence of low-frequency types may be imprecise, but in general, the sample size should be enough to maintain a good power and precision for analysis.

Socio-demographic characteristics, behavioral, TB history, and co-infection data were collected from eligible study participants using a pre-tested and close-ended structured questionnaire to avoid the interviewer's information bias. However, as the study's nature, the cross-sectional study design is compounded by recall bias, which might be unavoidable biases in certain independent variables, including the duration of illness where we observed several participants did not respond for it. To minimize recall bias, we excluded the participants that did not respond to the specific variable. Data with missed responses by some respondents were traced back to the participants and, if possible, corrected. A few data that were not very important and missed by most respondents were omitted from the analysis. If some participants missed important data and could

not complete it by tracing back, they included only the respondents for the analysis. Samples were collected from eligible study participants consecutively until the required sample size was obtained from the study sites. The technique used for recruiting study participants could have avoided the bias that can occur by selection bias. However, certain biases may occur by chance, as seen in the protective effect of alcohol consumption on the development of MDR-TB. This was thus, excluded from the main conclusions.

Before statistical analysis, data were validated for accurateness. The confounding effect of independent variables was avoided by analyzing the data using multivariable logistic regression. Model fit of logistic models were assessed using the Hosmer-Lemeshow test and a visual examination using the sensitivity/specificity and receiver operating characteristic curves. We found no major problem with the statistical models used.

External validity

We were able to sample from a large part of Tigray in a relatively long period, but some areas may be over-or under-sampled. As the samples were collected based on the stated criteria from study participants from the general population, we believe that the findings represent the real situation in Tigray Region. In general, the internal validity was sufficient to warrant the conclusions we have drawn from the study results. While patterns differ between Ethiopia regions, our studies bring interesting results to improve TB control in Ethiopia in general. Thus, we hope that the external validity of the studies is at a relevant level.

Conclusions and Recommendations

WGS analysis uncovered the presence of diversified Mtb genotypes circulating in the Tigray Region with the highest proportion of L4. The high proportion of MDR-TB among new and previously treated patients and the association of L4 with drug-resistance is very alarming, which calls for an urgent need for locally adapted intervention. The high proportion of MDR-TB among newly diagnosed cases and the high level of RTI indicate an ongoing transmission, which suggests the need for an enhanced TB control program performance to interrupt transmission. The increased proportion of MDR-TB among previously treated cases indicates a need for better patient management to help prevent the evolution of drug resistance.

The study highlighted the usefulness of mutations at *rpoB*, *katG*, *embB*, *rps*L, *pncA*, *ethA*, *gyr*A and *rrs* genes as a molecular marker for the rapid detection of resistance to RIF, INH, ETB, SM, PZA, ETH, FLQs and injectable 2nd-line anti-TB drugs, respectively. Hence, rapid detection and treatment of DR-TB can significantly improve the TB control program to halt transmission. WGS analysis showed numerous mutations that cause resistance to all 1st-and 2nd-line anti-TB drugs that are not included in the WHO and nationally recommended molecular assays. A significant proportion of disputed *rpoB* mutations were reported.

Overall, the findings underlined the importance of urgently strengthening the regional TB control program to detect and provide appropriate early treatment and follow-up for TB cases. There should be an optimal implementation of the five WHO-recommended priority actions to manage DR-TB to reduce the current high MDR-TB burden in the study region. Additionally, our findings suggest the need for conducting periodic surveillance of drug-resistance conferring mutations, early diagnosis and treatment of TB. Scale-up of rapid testing and detection of MDR/RR-TB cases in the region is crucial. Besides, given the limitations of the currently recommended molecular techniques (LPA) for detecting drug resistance, the NTCP should consider establishing WGS at regional levels so that 1st- and 2nd-line DR mutations can periodically be monitored to prevent and control the transmission of DR-TB in the community.

Future works

Our study indicates a high proportion of MDR-TB among new cases, which shows the active transmission of DR-TB in the community. This suggests the need for further study on household contacts to assess the transmission dynamics of DR-TB in the community. Understanding the drug

susceptibility patterns and associated factors of Mtb among extrapulmonary patients is an area for future studies. This has not yet been done and necessitates future work in this study area to have full information on DR-TB's magnitude in the study area.

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Appendix I: Enclosed papers

PAPER I

PLOS ONE

RESEARCH ARTICLE

Characteristics of pulmonary multidrug-resistant tuberculosis patients in Tigray Region, Ethiopia: A cross-sectional study

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Abstract

Background

Tuberculosis (TB) is among the top 10 causes of mortality and the first killer among infectious diseases worldwide. One of the factors fuelling the TB epidemic is the global rise of multidrug resistant TB (MDR-TB). The aim of this study was to determine the magnitude and factors associated with MDR-TB in the Tigray Region, Ethiopia.

Method

This study employed a facility-based cross-sectional study design, which was conducted between July 2018 and August 2019. The inclusion criteria for the study participants were GeneXpert-positive who were not under treatment for TB, PTB patients' ≥15 years of age and who provided written informed consent. A total of 300 participants were enrolled in the study, with a structured questionnaire used to collect data on clinical, sociodemographic and behavioral factors. Sputum samples were collected and processed for acid-fast bacilli staining, culture and drug susceptibility testing. Drug susceptibility testing was performed using a line probe assay. Logistic regression was used to analyze associations between outcome and predictor variables.

Results

The overall proportion of MDR-TB was 16.7% (11.6% and 32.7% for new and previously treated patients, respectively). Of the total MDR-TB isolates, 5.3% were pre-XDR-TB. The proportion of MDR-TB/HIV co-infection was 21.1%. A previous history of TB treatment AOR 3.75; 95% CI (0.7–2.24), cigarette smoking AOR 6.09; CI (1.65–2.50) and patients who had

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Abbreviations: DR-TB, drug resistant tuberculosis; FMOH, Federal Ministry of Health of Ethiopia; INH, Isoniazid; LPA, Line probe assay; MDR-TB, Multidrug-resistant tuberculosis; MTB, *Mycobacterium tuberculosis*; Pre-XDR-TB, Pre-extensively drug-resistant tuberculosis; PTB, Pulmonary tuberculosis; RIF, Rifampicin; WHO, World Health Organization.

an intermittent fever (AOR = 2.54, 95% CI = 1.21–5.4) were strongly associated with MDR-TB development.

Conclusions

The magnitude of MDR-TB observed among new and previously treated patients is very alarming, which calls for an urgent need for intervention. The high proportion of MDR-TB among newly diagnosed cases indicates ongoing transmission, which suggests the need for enhanced TB control program performance to interrupt transmission. The increased proportion of MDR-TB among previously treated cases indicates a need for better patient management to prevent the evolution of drug resistance. Assessing the TB control program performance gaps and an optimal implementation of the WHO recommended priority actions for the management of drug-resistant TB, is imperative to help reduce the current high MDR-TB burden in the study region.

Introduction

Tuberculosis (TB) is a chronic infectious disease, which is most commonly caused by *Mycobacterium tuberculosis (*MTB). TB has continued to be a major global public health concern, being one of the top 10 leading causes of death and the top killer among infectious diseases [1]. It is also the leading cause of death among people living with HIV/AIDS and the main cause of antimicrobial resistance-associated death [2]. Globally, there were an estimated 10 million new TB cases and 1.2 million deaths from TB in 2018 [3].

One of the major factors fuelling the TB epidemic is the emergence and spread of drug-resistant (DR) strains of MTB on new and previously treated cases, which creates a threatening and challenging condition for the prevention and control of TB [4]. Out of the total TB incident cases reported in 2018, 484,000 were resistant to rifampicin (RR-TB), and of these, 78% (3.4% new cases and 18% previously treated cases) had multidrug-resistant TB (MDR-TB). A total of 214,000 patients died due to MDR/RR-TB in 2018 [3].

TB is the most common cause of morbidity and mortality in Ethiopia. The country is among the three highest TB, TB/HIV and MDR-TB burden countries with estimates of 165,000 new TB cases and a rate of 151/100,000 population reported in 2018. In the same year, the number of cases of MDR/RR-TB was 1,600, the number of fatalities from TB was 24,000 for HIV-negative people and an additional 2,200 when including people living with HIV/AIDS [3].

Several studies in Ethiopia assessed the magnitude of drug-resistant TB. A review done by Biadglegne *et al.* reported that the occurrence of MDR-TB among TB patients in Ethiopia ranged from 3.3% to 46.3% [5]. Moreover, based on a recent meta-analysis report, the pooled estimate of MDR-TB among new and previously treated cases was 2% (1 to 2%) and 15% (12 to 17%), respectively [6]. Another study reported a MDR-TB prevalence ranging from 0 to 46.3% [7].

Previous studies in the different Regions of Ethiopia have shown variabilities in the magnitude of MDR-TB. The Oromia Region had 33.2% of MDR-TB cases [8], while in Jigjiga 10.2% of those smear-positive were MDR-TB patients [9]. In studies done in the Amhara Region, southwest Ethiopia and Addis Ababa, the magnitude of MDR-TB was reported to be 36.3%, 27.7% and 39.4%, respectively [10–12].

Early case detection and treatment of TB cases is essential to prevent and control drug-resistant TB. In the Tigray Region where this study was conducted, the treatment success rate was 80%, which is lower than the national and WHO target of achieving a 90% treatment success rate among the detected smear-positive cases. The treatment success rate observed in the Tigray Region was also lower than other regions such as the Afar Region in Ethiopia, which recorded a treatment success rate of 89% [13]. The major factor leading to a poor treatment success rate is treatment failure, which is mostly caused by drug-resistant (DR-TB) strains [13, 14].

In the Tigray Region, there is limited to no data on the burden and associated factors of DR-TB. Therefore, this study aimed at assessing the drug susceptibility pattern of TB for the first- and second-line anti-TB drugs and associated factors among TB patients in the Tigray Region, Ethiopia.

Material and methods

Study area

The study was conducted in six hospitals: the Alamata Hospital, Southern Zone; the Mekelle Hospital, Mekelle Special Zone; the Adigrat Hospital, Eastern Zone; the Adwa Hospital, Central Zone; the Shire/Suhul Hospital, Northwestern Zone and the Humera/Kahsay Abera Hospital, Western Zone) of the Tigray Regional State. The region has an estimated total population of 5.13 million [15]. The region is administratively divided into 7 zones (one especial zone, Mekelle), 52 districts and 814 *Kebeles* (lowest administrative unit). The health infrastructure of the region includes 40 hospitals, 223 health centers and 710 health posts serving the population of Tigray and neighboring regions. All the hospitals and health centers are equipped with TB diagnostic facilities (acid-fast bacteria (AFB) smear microscopy) and some hospitals with additional GeneXpert TB diagnostic facility. The Tigray Health Research Institute (THRI) is the only facility providing TB culture and drug susceptibility testing in the Region. The Stop TB (Directly Observed Treatment Short-course (DOTS)) Strategy is being implemented to control TB in the Region. The DOTS-Plus program has been introduced in all health facilities. Currently, there are nine treatment initiation centers and 62 treatment follow-up centers for MDR-TB in the Region. Fig 1 provides the geographical information about the hospitals and zones of the study area.

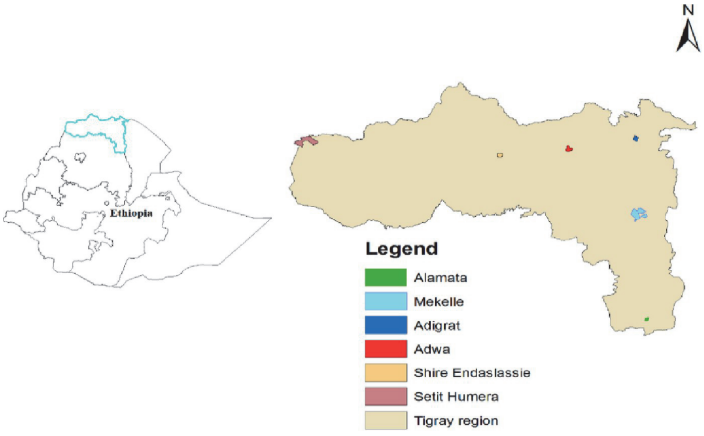

Fig 1. Map of the study area in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

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Study design, population and inclusion criteria

The study was a hospital-based, cross-sectional study conducted from July 2018 to August 2019. The source population consisted of all presumptive pulmonary TB (PTB) cases in the Tigray Region during the study period. The study population were all presumptive PTB cases who visited the selected health facilities in the region during the study period. The inclusion criteria included PTB patients' who were not under treatment during the study, ≥15 years of age, who were GeneXpert-positive and could provide written informed consent, were included in the study. Critically ill patients from whom sociodemographic, clinical data and sputum samples could not be obtained, patients <15 years of age and extra-pulmonary TB cases were excluded from the study.

Sample size determination

The sample size was determined by taking the required minimum number of MDR-TB patients to be enrolled in the study, which was estimated to be between 30–40 cases. This was to establish a sample with sufficient number of MDR-TB isolates to allow a sufficient power to find different varieties of MDR-TB isolates. Based upon an expected level of 10% [9] MDR-TB among TB patients, we aimed at recruiting at least 300 TB patients for the study. This would allow us to sample enough patients for the molecular studies, and also maintain a reasonable level of precision for the prevalence estimates. Without adjusting for clustering, the precision of the estimate would be at 6.7%-14.2% (95% CI, a relative precision of ± 3.3%).

Six government hospitals from the Tigray Region were selected based on the availability of the GeneXpert diagnostic technique as the primary test for presumptive PTB patients from the main zones of the Region without considering other factors (risk of high MDR-TB, high HIV and historically poor TB control program, etc.). The selected hospitals were the only hospitals with the GeneXpert test for the diagnosis of all presumptive PTB patients during the study. These hospitals were also giving diagnosis services for other neighboring health institutes that do not have a GeneXpert test. The standard practice of the hospitals was, first testing of all presumptive PTB patients by GeneXpert test, if the GeneXpert test result is susceptible TB the patient immediately started on 1st line-TB treatment and if the result is RIF-R TB the patient linked to MDR-TB clinic and immediately sputum sample used to be collected for culture before starting MDR-TB treatment.

However, for the purpose of this study, sputum samples were collected for culture and drug susceptibility testing from all consecutive GeneXpert positive PTB patients who fulfilled the inclusion criteria. A consecutive sampling technique was employed to recruit the study sample in all the hospitals until the required sample size was obtained.

Operational definitions of variables

New case: A patient who has never had treatment for TB, or has been on treatment for less than four weeks.

Previous treatment: A patient who took TB treatment for one month or more in a previous time.

Failure after treatment patient: A patient who has previously been treated for TB, and whose treatment failed at the end of their most recent course of treatment.

Defaulter (treatment after loss to follow-up): A patient whose anti-TB treatment was interrupted for two consecutive months or more.

Relapse: A patient who has previously been treated for TB, was declared cured or whose treatment was completed at the end of their most recent course of treatment and is now diagnosed with a recurrent episode of TB.

Mono-drug resistance: Resistance to only one of the four first-line anti-TB drugs.

Any drug resistance: Resistance to one or more first-line anti-TB drugs.

Multi-drug resistance: Resistance to at least both isoniazid and rifampicin.

Rifampicin resistance: Resistance to rifampicin detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs.

Primary resistance (resistance among new TB cases): Resistance in patients who did not have a history of anti-TB treatment.

Secondary resistance (resistance among previously treated cases): Resistance in patients previously treated with anti-TB drugs.

Treatment success rate refers to the percentage of notified TB patients who were successfully treated.

Data collection

Socio-demographic and clinical data were collected from patients using a pre-tested structured questionnaire: Age, sex, residence, number of people living in a single room, history of imprisonment, history of TB treatment, contact history with TB patients in the past two years, and TB symptoms like cough, blood in sputum, fever, chest pain, shortness of breath, fatigue, night sweats, loss of appetite and body weight loss. Data on the history of previous medical illnesses and behavioral factors, including alcohol intake, cigarette smoking and Khat chewing habits, were also collected. HIV counselling and testing were performed for TB patients based on the recommendations of the Federal Ministry of Health of Ethiopia (FMOH) testing algorithm [16] at the study hospitals during the study period.

GeneXpert® MTB/RIF assay

From each patient, a 4 ml sputum sample was collected "on-the-spot" and treated with a sample reagent (SR) containing NaOH and isopropanol according to the recommendation of FMOH [17]. The SR was added in a 2 to 1 ratio of the sputum sample, which was then homogenized and incubated for 15 minutes at room temperature following the manufacturer's instructions (Cepheid, Sunnyvale, CA, USA) [18]. The treated samples were transferred into the cartridge, and the cartridge was loaded into the GeneXpert instrument. Moreover, the Xpert® MTB/RIF purifies and concentrates MTB from the sputum samples, isolates genomic material from the captured mycobacteria by sonication, and subsequently amplifies the genomic DNA by PCR. The process identifies MTB DNA and rifampicin resistance, thus inducing mutations in the RNA polymerase beta (rpoB) gene in the MTB genome in a real-time format using fluorescent probes called molecular beacons.

Sputum collection for culturing

A 5–10 ml sputum sample was collected from every GeneXpert MTB/RIF assay-positive participant by laboratory personnel, using a coded and sterile 50 ml falcon tube according to the recommendation of FMOH [17].

All sampled sputa for each participant were properly packed and kept at 4˚C for transportation in an ice bag to the Tigray Health Research Institute (THRI), according to the international standards of the WHO recommendation for transport of a biological substance; category B, UN-3373. Specimens arrived within four-five days of collection, and were processed within seven days from the time of first collection.

Culture and identification

Decontamination and sputum processing. Sputum samples were digested using freshly prepared N-acetyl-L-cysteine (NALC) and decontaminated by NaOH (1%). Phosphate buffer

(PH 6.8) were added to neutralize NaOH, and dilutes the homogenate to lessen the viscosity and specific gravity. The homogenate was centrifuged at 3000g at 4°C for 15 minutes [19]. The direct microscopic examination of sputum, and from culture for acid-fast bacteria (AFB) using the standard Ziehl-Neelsen staining, was done at THRI.

Sputum culture. The decontaminated supernatant decanted sputum sample was cultured on a Lowenstein-Jensen (LJ) egg medium and on a liquid culture Mycobacterium Growth Indicator Tube; BACTEC MGIT 960 culture (Becton Dickinson Microbiology systems, Sparks, MD, USA), following the standard operational procedures. The tubes for the solid culture were incubated at 37˚C in a slant position to ensure an even distribution of inoculums for one week and thereafter at 37˚C in air for another seven weeks, and then checked once a week for mycobacterial growth. Cultures were considered negative when no colonies or growth were seen after eight weeks of incubation for a solid culture and six weeks (42 days) for a liquid culture. The growth of mycobacteria were confirmed by its typical colony morphology, acid-fast bacilli (AFB) staining, Capilia antigen test and inoculation onto a blood agar plate to rule out contamination.

Drug susceptibility testing for first-line and second-line anti-TB drugs using Line Probe Assay (LPA)

Drug-susceptibility testing for first-line anti-TB drugs (isoniazid and rifampicin) using GenoType® MTBDRplus, and second line anti-TB drugs (ofloxacin, levofloxacin, moxifloxacin, amikacin, capromycin, kanamycin and viomycin) using GenoType MTBSL, was carried out by line probe assay genotypic method following the manufacturer's instruction (GenoType® MTBC; Hain Life Science, Nehren, Germany).

DNA extraction form culture was done using GenoLyse® kit (A and B) in a DNA contaminating free working area. After centrifugation, the supernatant was transferred to a new cryotube. In another room free from contaminating DNA, amplification Mixes A (10 μl) and B (35 μl) were prepared freshly and a total of 45 μl Master Mix was transferred to each polymerase chain reaction (PCR) tubes. The DNA extract (5 μl DNA) was added to respective PCR tubes, and 5 μl of DNA extract from H37Rv quality control strain to the positive control tube and 5 μl of distilled water to negative control tube was added. After amplification, the amplicon was detected with a series of procedures by adding different reagents to the strip. The strips formed color bands after the addition of the final substrate reagent [20].

Quality assurance and quality control

All laboratory analyses were carried out following standard operating procedures. Both the solid culture and LPA procedures were checked and validated. Reference strains of MTB H37Rv were used as quality control organisms throughout the LPA test. Moreover, both the start and end controls were used during each batch of specimen processing and DNA extraction, as well as no template control being used for LPA reagents.

Data entry and statistical analysis

Data were double-checked for completeness and cleaned before entry. A data missed by some respondents were traced back to the participants and completed the missed data. A few data which were not very important and missed by most respondents were omitted from the analysis as a whole. If important data were missed by some participants and we could not complete it by tracing back, we included only the respondents in the analysis. The complete and cleaned data were entered using the Epidata 3.1 data entry software. After cleaning and validation, the data were transferred into Stata (Stata SE 15/ SE for Windows, StataCorp, College Station, TX)

software for further statistical analysis. The study participants were categorized into two groups: patients with non-MDR-TB and patients with MDR-TB. Descriptive statistics were computed, and frequencies and proportions were presented in tables. Further statistical analyses were performed using a univariable and multivariable logistic regression to identify associations between sociodemographic, behavioral and clinical factors with the main outcome variable (MDR-TB). Results from the logistic regression analysis were presented using Odds Ratios (OR), with 95% Confidence Intervals and p-values. Variables with a $p < 0.20$ in the univariable analyses were included in the multivariable models. Models were built using a backward selection principle, with a likelihood-ratio (LR) test of $p = 0.10$ as a cut-of-point for excluding variables. A model fit was assessed using the Hosmer-Lemeshow test and a graphical examination using the sensitivity/specificity and receiver operating characteristic (ROC) curves.

Ethical considerations

Ethical clearance was obtained from Mekelle University, College of Health Sciences Ethical Review and Research Committee (ERC 1438/2018), Ministry of Science and Higher Education, Ethiopia (SHE/S.M/14.4/708/19) and the Regional Committee for Medical Research Ethics in Eastern Norway (REK Øst) (2018/1118/REK sør-øst A). Written informed consent was secured from all study participants before the commencement of the study.

Results

A total of 6322 presumptive PTB patients were excluded from the study because of the exclusion criteria stated. Primarily they were GeneXpert negative. In the present study, 300 GeneXpert® MTB/RIF assay positive study participants were included. Of these, 227 (75.67%), six (2%), 60 (20%) and seven (2.3%) were MTB culture-positive, non-*Tuberculosis mycobacterium (*NTM), culture-negative and contaminated, respectively. In the rest of this paper, only the 227 study participants with culture-positive tests were used for further analysis. The flow of the study participants' recruitment process is depicted in Fig 2.

The median age of all 300 participants was 30 years, ranging from 15–85 years. Of these, 196 (65.3%) were males and 104 (34.7%) females. A total of 220 (73.3%) participants were new TB cases, and 80 (26.7%) were previously treated patients. Furthermore, a majority of the participants 112 (37.3%) were in the age group from 25–34 years, 140 (46.7%) were married, 167 (55.7%) were urban dwellers, 98 (32.7%) had only elementary school, 84 (28.0%) were self-employed and 95 (31.7%) had no monthly income.

Drug resistance patterns of first- and second-line anti-TB drugs

As presented in Table 1, a total of 40 (17.6%) of the 227 isolates were resistant to RIF by GenoType MTBDRplus assay while 42 (18.5%) were resistant to RIF by Xpert® MTB/RIF assay. Moreover, six (3.2%) RIF resistant isolates using Xpert® MTB/RIF assay were non MDR-TB by the Geno Type MTBDRplus method, and 2/38 (5.3%) RIF susceptible by Xpert® MTB/RIF assay were MDR-TB using the Geno Type MTBDRplus method.

Table 2 shows the pattern of resistance among 227 MTB isolates. The dominant isolates 189 (83.3%) were susceptible to INH and/or RIF, two (0.9%) had a mono resistance to RIF, three (1.3%) a mono resistance to INH, 43 (18.9%) a resistance to INH and/or RIF and 41 (18.1%) an overall resistance to INH. The proportion of MDR-TB among new and previously treated patients was 20 (11.6%) and 18 (32.7%), respectively, and the overall MDR-TB was 38 (16.7%). In this study, two (5.3%) of the MDR-TB isolates were pre-XDR-TB, which were resistant to fluoroquinolones (FQs), one of the second-line anti TB drugs. However, for the rest of second-line anti-TB drugs, the MDR-TB isolates were susceptible.

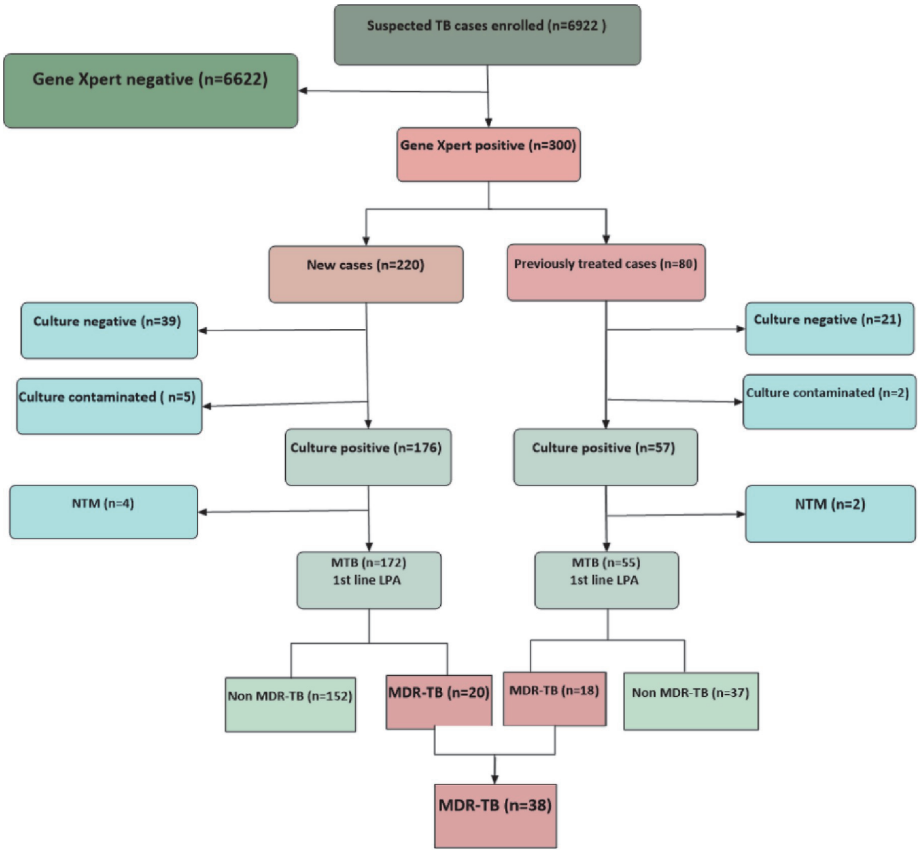

Fig 2. Flow of the procedure followed for all patients recruited in the study in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

https://doi.org/10.1371/journal.pone.0236362.g002

The proportion of overall TB/HIV, non-MDR-TB/HIV and MDR-TB/HIV co-infection among the 227 TB patients was 41 (33%), 33 (17.5%) and eight (21.1%), respectively. The Zonal distribution showed that MDR-TB was highest in the Southern Zone (21.1%), followed by the Mekelle Special Zone (18.4%) (Fig 3).

Table 1. Rifampicin resistance patterns of isolates based on MTB status category of study participants in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

	Rifampicin resistance pattern			
	Genotype MTBDR plus			
	Non MDR-TB (n = 189)			MDR-TB (n = 38)
		Susceptible, F (%)	Resistant (%)	Resistant (%)
GeneXpert	Susceptible	183 (96.8)	0	2 (5.3)
	Resistant	4 (2.1)	2 (1.1)	36 (94.7)

https://doi.org/10.1371/journal.pone.0236362.t001

Table 2. Drug resistance patterns of isolates to first- and second-line anti-TB drugs with TB patient category of study participants in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

First-line resistance pattern	New cases (n = 172), F (%)		Previously treated cases (n = 55), F (%)	Total cases (n = 227), F (%)
Any-S	152 (88.4)		37 (67.3)	189 (83.3)
RIF-R	21 (12.2)		19 (34.5)	40 (17.6)
RIF mono- R	1 (0.6)		1 (1.8)	2 (0.9)
RIF-S	151 (87.8)		36 (65.5)	187 (82.4)
INH-R	23 (13.4)		18 (32.7)	41 (18.1)
INH mono-R	3 (1.7)		0 (0)	3 (1.3)
INH-S	149 (86.6)		37 (67.3)	186 (81.9)
Any-R	24 (14)		19 (34.5)	43 (18.9)
MDR	20 (11.6)		18 (32.7)	38 (16.7)
Second-line resistance pattern (38)	**New cases (20), F (%)**		**Previously treated cases (18), F (%)**	**Total cases (38), F (%)**
FLQ	R	0	2 (11.1)	2 (5.3)
	S	20 (100)	16 (88.9)	36 (94.7)
AMK, CAP, KAN	R	0	0	0
	S	20 (100)	18 (100)	38 (100)
KAN, CAP, VIO	R	0	0	0
	S	20 (100)	18 (100)	38 (100)
KAN, AMK, CAP, VIO	R	0	0	0
	S	20 (100)	18 (100)	38 (100)
Low-level KAN	R	0	0	0
	S	20 (100)	18 (100)	38 (100)

R = resistant, S = susceptible, FLQ = Fluoroquinolones (Ofloxacin, levofloxacin, Moxifloxacin), AMK = Amikacin, KAN = kanamycin, CAP = Capromycin, VIO = Viomycin.

https://doi.org/10.1371/journal.pone.0236362.t002

Factors associated with MDR-TB

Patients' sociodemographic parameters, risk behaviors, MDR-TB/HIV co-infection status, contact history and clinical presentations were compared between non-MDR and MDR-TB cases using univariable and multi-variable logistic regression analysis (Tables 3–5).

A history of previous TB treatment showed a strong association (AOR = 4.26, 95% CI = 1.99–9.14) with MDR-TB development, and patients with cigarette smoking habits (AOR = 6.09, 95% CI = 1.65–22.50) were more likely to develop MDR-TB compared to those who did not smoke. Patients who had an intermittent fever (AOR = 2.54, 95% CI = 1.21–5.4) were two times more likely to develop MDR-TB compared to those who did not experience a fever. Surprisingly, patients who used to consume alcohol (AOR = 0.28, 95% CI = 0.10–0.77) were less likely to develop MDR-TB compared to those who did not take alcohol.

No sociodemographic characteristics and co-infections status were associated with MDR-TB development. This included an absence of association between MDR-TB and HIV infection. In the univariable analysis, patients with a duration of symptoms > 60 days were more likely to develop MDR-TB, but this variable did not show a significant association in the multivariable model.

Discussion

This is the first and largest study attempting to assess the magnitude and associated factors of MDR-TB in the Tigray Region of Ethiopia. A periodic assessment of the prevalence and associated factors of drug resistance in high TB burden countries is essential to identify early- and

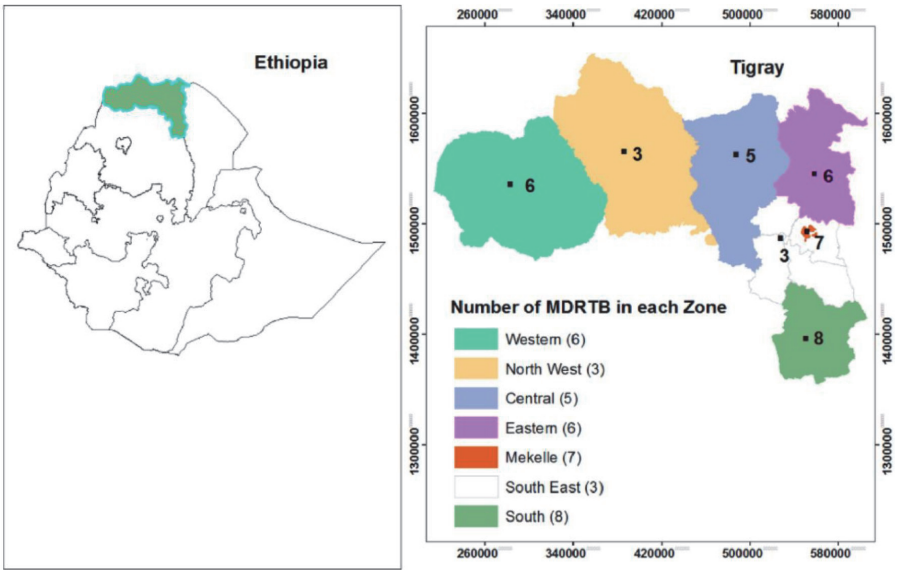

Fig 3. Zonal distribution of 38 MDR-TB among TB patients (N = 227) in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

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address the challenges of drug-resistant TB transmission. This helps to enhance the TB control program performance and achieve the End TB Strategy goals.

The overall proportion of MDR-TB observed in our study was 16.2% (11.6% among new cases and 32.7% among previously treated cases). The WHO data indicates that 6–10% of new TB cases and 13–60% of cases in previously treated patients are MDR [21]. The overall proportion of MDR-TB observed in our study was higher compared to previous studies conducted in Addis Ababa, Ethiopia [22, 23], northwest Ethiopia [24, 25], Jigjiga in the Somali Region [9] and the Oromia Region [26], which showed an overall prevalence of MDR-TB of 11.5%, 5.6%, 10.2%, 1.8%, 1.8% and 4.7%, respectively. The finding was also higher than the study done in China [27], Kenya [28], Tanzania [29], India [30], Vietnam [31], Lima Peru [32], Dalian China [33] and northeastern China [34], where the overall MDR-TB prevalence was estimated at 11.3%, 4.8%, 6.3%, 5.6%, 6.9%, 6.6%, 10.1%, 8.7%, respectively. Conversely, the overall MDR-TB proportion observed in our study was lower than former study findings, which showed a 39.4%, 27.2%, 39.2% and 26% overall MDR-TB prevalence reported from Addis Ababa, Ethiopia [12], southwest Ethiopia [10], Uganda [35] and Taiwan [36], respectively. The increased proportion of MDR-TB observed in our study is very alarming to the TB/MDR-TB control program in the study area, as well as for the country at large.

Increased MDR-TB among new TB cases is an indicator of the ongoing transmission of drug-resistant TB. The proportion of new MDR-TB (11.6%) cases observed in our study is higher than many other studies conducted in Ethiopia, such as eastern Ethiopia (1.1%) [37], northwest Ethiopia (2.3%) [24], Jigjiga, in the Somali Region (4.5%) [9] and the Amhara Region (1.0%) [38]. The finding is also higher than studies conducted in other countries: Tanzania (4.3%) [29], India (2.9%) [30], Vietnam (4.2%) [31], Dalian China (5.8%) [33] and

Table 3. Sociodemographic and behavior factors associated with MDR-TB among the study participants in the Tigray region, northern Ethiopia, July 2018 to August 2019.

Variables	Non-MDR (189), F (%)	MDR (38), F (%)	COR (95% Cl)	P-value	AOR (95% Cl)	P-value
Sex						
Male	122 (64.6%)	22 (57.9)	1		-	-
Female	67 (35.5)	16 (42.1)	1.32 (0.65–2.69)	0.438		
Age in years						
15–24	38 (20.1)	9 (23.7)	1			
25–34	76 (40.2)	14 (36.8)	0.78 (0.3–1.96)	0.594	-	-
35–44	36 (19.1)	10 (26.3)	1.17 (0.43–3.22)	0.757		
45–54	20 (10.6)	2 (5.3)	0.42 (0.08–2.14)	0.298		
≥55	19 (10.1)	3 (7.9)	0.67 (0.16–2.75)	0.575		
Residence						
Urban	103 (54.5)	24 (63.2)	1			
Rural	86 (45.5)	14 (36.8)	0.7 (0.34–1.43)	0.328	-	-
Marital status						
Single	90 (47.6)	15 (39.5)	1			
Married	86 (45.5)	17 (44.7)	1.18 (0.56–2.52)	0.658		
Divorced	4 (2.1)	2 (5.3)	3 (0.50–17.85)	0.227	-	-
Widowed	9 (4.8)	4 (10.5)	2.67 (0.73–9.77)	0.139		
Pregnant (48)						
No	8 (80)	37 (97.4)	1			
Yes	2 (20)	1 (2.6)	2.53 (0.22–28.6)	0.454	-	-
Lactating (48)						
No	9 (90)	37 (97.4)	1			
Yes	1 (10)	1 (2.6)	5.08 (0.31–3.07)	0.254	-	-
Family size						
≤3	109 (57.7)	19 (50)	1			
≥4	80 (42.3)	19 (50)	1.36 (0.68–2.74)	0.385	-	-
Education						
Illiterate	60 (31.8)	10 (26.3)	1			
1–8 grades	58 (30.7)	16 (42.1)	1.66 (0.69–3.95)	0.256	-	-
9–12 grades	54 (28.6)	8 (21.1)	0.89 (0.33–2.42)	0.817		
Diploma and above	17 (9)	4 (10.5)	1.41 (0.39–5.07)	0.597		
Occupation						
Housewife	20 (10.6)	5 (13.2)	1			
Farmer	46 (24.3)	10 (26.3)	0.87 (0.26 2.87)	0.819	-	-
Self-employed	58 (30.7)	8 (21.1)	0.55 (0.16–1.88)	0.342		
Government employee	10 (5.3)	2 (5.3)	0.8 (0.13–4.87)	0.809		
Student	13 (6.9)	4 (10.5)	1.23 (0.28–5.45)	0.785		
No work	42 (22.2)	9 (23.7)	0.86 (0.25–2.89)	0.804		
Monthly income (Birr)						
<500	41 (21.7)	11 (29)	1			
500–2000	48 (25.4)	10 (26.3)	0.78 (0.3–2.01)	0.603		
>2000	39 (20.6)	4 (10.5)	0.38 (0.11–1.30)	0.124	-	-
No means of income	61 (32.3)	13 (34.2)	0.79 (0.32–1.94)	0.614		
Smoking						
No	176 (93.1)	32 (84.2)	1		1	
Yes	13 (6.9)	6 (15.8)	2.54 (0.9–7.17)	0.079	6.09 (1.7–22.5)	**0.007**

(*Continued*)

Table 3. (Continued)

Variables	Non-MDR (189), F (%)	MDR (38), F (%)	COR (95% Cl)	P-value	AOR (95% Cl)	P-value
Alcohol intake						
No	111 (58.7)	28 (73.7)	1		1	
Yes	78 (41.3)	10 (26.3)	0.51 (0.23–1.11)	0.088	0.28 (0.1–0.77)	**0.014**
Khat chewing						
No	173 (91.5)	33 (86.8)	1			
Yes	16 (8.5)	5 (13.2)	1.64 (0.56–4.78)	0.366	-	-

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northeastern China (4.2%) [34]. The high proportion of MDR-TB among newly diagnosed TB patients observed in this study is of high concern, which requires an urgent intervention to improve the quality of TB control to interrupt the transmission of drug-resistant TB [39].

The proportion of MDR-TB (32.7%) among previously treated cases in our study was more or less similar with a study report from southwest Ethiopia [40], which showed an MDR prevalence of 31.4%. However, our report was lower than that reported from the St. Peter's TB Specialized Hospital, Addis Ababa, Ethiopia, which showed an MDR-TB prevalence of 58% [41]. This may not be surprising as the St-Peter's Specialized Hospital in Addis Ababa is the national referral hospital for specialized TB care in the country. Conversely, our finding was higher than several other previous study results reported from Jigjiga, the Somali Region, Ethiopia [9], Dalian China [33], northeastern China [34] and Vietnam [31], which showed an MDR prevalence of 22.6%, 17.7%, 27.6% and 23.1%, respectively. The increased MDR-TB proportion among previously treated cases in our study indicates a need for better patient management to help prevent the evolution of resistance in the study area.

Five percent of the cases in our study had pre-XDR-TB or FQs-resistant MDR-TB, which is higher than a recent study conducted in Ethiopia (3.4%) [42] and China (0.7%) [34]. Studies in

Table 4. Associations of TB/HIV co-infection and contact history with MDR-TB among study participants in the Tigray region, northern Ethiopia, July 2018 to August 2019.

Variables	Non-MDR-TB (%) (n = 189)	MDR-TB (%) (n = 38)	OR (95% Cl)	P-value
TB patient history in family				
No	144 (76.2)	26 (68.4)	1	
Yes	45 (23.8)	12 (31.6)	1.48 (0.69–3.16)	0.316
History of TB close contact				
No	149 (78.8)	25 (65.8)	1	
Yes	40 (21.2)	13 (34.2)	1.94 (0.91–4.12)	0.086
History of MDR-TB contact				
No	176 (93.1)	33 (86.8)	1	
Yes	13 (6.9)	5 (13.2)	2.05 (0.69–6.14)	0.199
Diabetes mellitus status				
No	183 (96.8)	36 (94.7)	1	
Yes	6 (3.2)	2 (5.3)	1.69 (0.33–8.73)	0.528
HIV status				
Negative	156 (82.5)	30 (79)	1	
Positive	33 (17.5)	8 (21)	1.26 (0.53–3)	0.600
History of prison				
No	163 (86.2)	34 (89.5)	1	
Yes	26 (13.8)	4 (10.5)	0.74 (0.24–2.25)	0.593

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Table 5. Associations of clinical presentations with MDR-TB among study participants in the Tigray Region, northern Ethiopia, July 2018 to August 2019.

Variables	Non-MDR-TB (189), F (%)	MDR-TB (%) (38), F (%)	OR (95% CI)	P-value	AOR (95% CI)	P-value
TB history						
No	152 (80.4)	20 (52.6)	1		1	
Yes	37 (19.6)	18 (47.4)	3.7 (1.78–7.68)	**0.001**	4.26 (1.99–9.14)	**<0.001**
Duration of illness in days (198)						
≤60	104 (62.6)	10 (31.3)	1			
>60	62 (37.4)	22 (68.7)	3.69 (1.64–8.30)	**0.002**	-	-
Body mass index						
>18.5	53 (28)	8 (21)	1			
≤18.5	136 (72)	30 (79)	0.68 (0.29–1.59)	0.377	-	-
Weight loss						
No	18 (9.5)	6 (15.8)	1			-
Yes	171 (90.5)	32 (84.2)	0.56 (0.21–1.52)	0.257	-	
Chest pain						
No	57 (30.2)	7 (18.4)	1			-
Yes	132 (69.8)	31 (81.6)	1.91 (0.8–4.6)	0.147	-	
Coughing for > 2 weeks						
No	27 (14.3)	6 (15.8)	1			
Yes	162 (85.7)	32 (84.2)	0.89 (0.34–2.33)	0.810	-	-
Shortness of breath						
No	92 (48.7)	19 (50)	1			
Yes	97 (51.3)	19 (50)	0.95 (0.47–1.90)	0.882	-	-
Hemoptysis						
No	172 (91)	33 (86.8)	1			
Yes	17 (9)	5 (13.2)	1.53 (0.53–4.44)	0.431	-	-
Intermittent fever						
No	119 (63)	17 (44.7)	1		1	
Yes	70 (37)	21 (55.3)	2.1 (1.04–4.25)	**0.039**	2.54 (1.21–5.4)	**0.014**
Night sweats						
No	44 (23.3)	9 (23.7)	1			
Yes	145 (76.7)	29 (76.3)	0.98 (0.43–2.22)	0.957	-	-
Appetite loss						
No	68 (36)	9 (23.7)	1			
Yes	121 (64)	29 (76.3)	1.81 (0.81–4.05)	0.148	-	-
Fatigue and malaise						
No	142 (75.1)	30 (79)	1			
Yes	47 (24.9)	8 (21)	0.81 (0.35–1.88)	0.617	-	-

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other countries showed a very high proportion of pre XDR-TB compared to our study result: Morocco (22.2%) [43], China (14.5%) [27], Vietnam (17.9%) [31], India (27.6%) [44], western India (22%) [45] and Pakistan (38.7%) [46]. Some of the factors associated with the high pre-XDR prevalence in those countries were related to a widespread use of FQs without prescriptions for the treatment of new TB cases and other undiagnosed respiratory infections in the private sectors [31, 45], with most of the study participants being treatment failures and chronic cases [45]. Overall, the proportion of pre-XDR-TB observed in our study is of great concern, thereby indicating the possibility of an increasing resistance to second-line anti-TB drugs in the region.

Previous studies indicated that several factors such as a previous history of TB treatment (failures, defaulters and relapses), contact with MDR-TB patients and water pipe smoking [23, 46, 47] are all associated with the development of MDR-TB. In our study, patients with a previous history of TB treatment were more likely to develop MDR-TB, with similar findings reported from other studies in Ethiopia and several other countries [6, 36, 40, 48–52]. This association might be related to unsatisfactory/noncompliance by patients or clinicians to anti-TB treatment, a lack of supervision of treatment or poor quality of the DOTS program, improper drug regimens and an inadequate or irregular drug supply, which may potentiate genetic mutations in the bacteria, and can result in acquired drug resistance [48, 53–55].

Cigarette smoking was associated with MDR-TB. This is similar to other study findings [23, 25, 35]. Cigarette smoking directly causes ciliary dysfunction; this diminishes the immunity of individuals, which makes them prone to primary MDR-TB [56, 57].

A univariable analysis showed that patients who experienced TB symptoms for a duration of more than 60 days were more likely to develop MDR-TB compared to their counterparts. This finding is in line with studies and reports from several other countries [40, 48, 58]. One of the mechanisms of MTB drug resistance arises from spontaneous point mutations. Over time, these mutations accumulate, and acquired drug resistance may occur if diagnosis and treatment is not initiated early [59]. A delay in treatment can also result in an overgrowth of MTB (a high grade of sputum positivity), which causes a delayed sputum conversion during treatment, and in turn is associated with an acquired MDR-TB [58, 60, 61].

The current proportion of MDR-TB/HIV co-infection was 21.1%, which is lower than the findings in previous studies in northwest Ethiopia (28.6%) [24], Addis Ababa, Ethiopia (79.8%) [12] and southwestern Ethiopia (43.5%) [40]. Nevertheless, this is higher than the results reported from Mali (10.5%) [47], Thailand (4.8%) [58] and India (5.6%) [30]. HIV infection is the strongest risk factor for the development of active TB regardless of the type of MTB, whether drug-susceptible or primary drug-resistant, as it suppresses the immune system of the individuals [62]. In our study, HIV co-infection was not associated with the development of MDR-TB, which is similar to other findings [40, 47, 58], though some studies reported its association with MDR-TB [37, 38, 49]. A review by Wells *et al*. reported that there was no association between MDR-TB and HIV in several studies in Africa, Russia, Vietnam, India and other multi-country studies. However, this review reported that there was an association in a study done in the United States and in Ethiopia, but in this review the association was only with primary MDR-TB [62]. This review in particular reported that a specific genotype family of drug-resistant strains of MTB might play a role in transmission, especially among people living with HIV infection. The Beijing genotype family, which includes the "W" strain of MTB implicated in many MDR-TB outbreaks in the United States, is more virulent and is associated with an anti-TB drug resistance in specific geographic settings [62].

We were surprised to observe the association between the consumption of alcohol and a lower chance of acquiring MDR-TB. As far as our literature review is concerned, there is no study that supports our finding. Our hypothesis is that this may have happened by chance. Further study is warranted to understand the link between alcohol intake and drug resistance.

In the current study, none of the sociodemographic factors were statistically associated with MDR-TB development, which is similar with other study reports [24, 58, 63]. A recent similar study in the Tigray Region reported that age was marginally associated with MDR-TB [64]. Yet, this result could not be reproduced in our study. Sociodemographic variables such as educational status [65], age [49] and residence [26] were associated with MDR-TB in studies conducted in the various other regions of Ethiopia.

Ethiopia has initiated an innovative community health program (health extension program) in all its regional states, including Tigray, over the last 15 years. This initiative can play a

big role in increasing early case detection and an improved treatment success rate by improving service access to TB patients [66, 67]. Regardless of the priceless role of health extension workers in TB/MDR-TB prevention and the control program, a study conducted in the Tigray Regional State reported that only one-fourth (25%) of the health posts were working efficiently [68]. This may contribute to a poor TB control program performance at the community level, which leads to a poor case detection and treatment outcome, and may ultimately contribute to an increased number of treatment failure cases and the emergence and transmission of drug-resistant TB. A previous study showed that a high proportion of defaulters was reported in the Tigray Region compared to other regions of Ethiopia [13]. In addition, another study in Tigray reported a low level of knowledge about the cause of TB and the consequences of a poor treatment adherence to its treatment [69]. Besides, a recent assessment report in Tigray Region indicates that only 21.7% of household contacts were screened for TB by health extension workers [70], which are by far lower than the target stated by the national TB program to screen all household contacts [71] and WHO target of greater than 90% household contacts to be screened for TB [3].

Several factors have contributed to the development and transmission of drug-resistant TB. Genetic diversities of drug-resistant isolates might be attributable to some host and environmental factors besides strain evolution in different geographic regions [72–75]. Drug resistance in MTB arises at a low frequency of spontaneous chromosomal mutations and inconsistent drug supply, suboptimal prescription and poor patient adherence [73, 74]. In particular, patients who have a previous TB treatment history such as treatment failures, defaulters or relapse cases are at a greater risk of developing MDR-TB [5, 75, 76]. The WHO underscores the importance of the proper management of MDR-TB to help address its global threat to human beings. To help alleviate this threat, the WHO has recommended five priority actions, including the prevention of the development of MDR-TB through a high-quality treatment of drug-susceptible TB, expanding the rapid testing and detection of drug-resistant TB, thereby providing immediate access to effective treatment and proper care, the prevention of transmission through infection control and an increase in political commitment and financing. High TB-burden countries like Ethiopia should be committed to optimally implementing these actions to reduce the emergence and transmission of drug-resistant TB in Tigray and all other regions of the country.

Limitations of the study

One of the limitations of this study was that non-MDR-TB isolates were not tested for second-line anti-TB drugs, as other studies found that such types of isolates were highly resistant to second-line anti-TB drugs [44]. The other limitation was that extra-pulmonary patients were not included in the study. Hence, the authors recommend further study on the overall study of the drug susceptibility pattern of first- and second line anti-TB drugs for non-MDR and MDR-TB from pulmonary and extra-pulmonary patients, in order to obtain full information on the magnitude of DR-TB in the study area.

Conclusion

The magnitude of MDR-TB observed among new and previously treated patients is very alarming, which calls for an urgent need for intervention. The high proportion of MDR-TB among newly diagnosed cases indicates an ongoing transmission, which suggests the need for an enhanced TB control program performance to interrupt transmission. The increased proportion of MDR-TB among previously treated cases indicates a need for better patient management to help prevent the evolution of drug resistance. The associated factors of this study indicate the need for consideration of these predisposing factors in the prevention and

intervention program of the Region. Overall, the findings highlight the importance of strengthening the Regional TB Control Program to detect and provide early appropriate treatment and follow-up for TB cases. Assessing the TB control program performance gaps in the region, and an optimal implementation of the five WHO-recommended priority actions for the management of drug-resistant TB, is imperative to reduce the current high MDR-TB burden in the study region.

Supporting information

S1 Data.
(XLSX)

S1 File. Questionnaire.
(PDF)

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PAPER II

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Frequency and patterns of first- and second-line drug resistance-conferring mutations in *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in a cross-sectional study in Tigray Region, Ethiopia

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ABSTRACT

Objectives: Tuberculosis (TB) is a preventable and treatable infectious disease, but the continuing emergence and spread of multidrug-resistant TB is threatening global TB control efforts. This study aimed to describe the frequency and patterns of drug resistance-conferring mutations of *Mycobacterium tuberculosis* (MTB) isolates detected from pulmonary TB patients in Tigray Region, Ethiopia.
Methods: A cross-sectional study design was employed to collect sputum samples from pulmonary TB patients between July 2018 to August 2019. Culture and identification tests were done at Tigray Health Research Institute (THRI). Mutations conferring rifampicin (RIF), isoniazid (INH) and fluoroquinolone (FQ) resistance were determined in 227 MTB isolates using GenoType MTBDRplus and GenoType MTBDR*sl*.
Results: Mutations conferring resistance to RIF, INH and FQs were detected in 40/227 (17.6%), 41/227 (18.1%) and 2/38 (5.3%) MTB isolates, respectively. The majority of mutations for RIF, INH and FQs occurred at codons *rpoB* S531L (70%), *katG* S315T (78%) and *gyrA* D94Y/N (100%), respectively. This study revealed a significant number of unknown mutations in the *rpoB*, *katG* and *inhA* genes.
Conclusion: High rates of mutations conferring resistance to RIF, INH and FQs were observed in this study. A large number of isolates showed unknown mutations, which require further DNA sequencing analysis. Periodic drug resistance surveillance and scaling-up of drug resistance testing facilities are imperative to prevent the transmission of drug-resistant TB in the community.

1. Introduction

Tuberculosis (TB) is a preventable and treatable infectious disease. However, the continuing emergence and spread of multidrug-resistant TB (MDR-TB) is threatening global TB control efforts. Among the 10 million incident TB cases reported in 2018, some 484 000 patients had rifampicin-resistant TB (RR-TB), and of these 78% were MDR-TB, defined as resistance to at least isoniazid (INH) and rifampicin (RIF), the two most potent first-line anti-TB

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drugs [1]. Among the reported MDR-TB cases in 2018, 6.2% were estimated to be extensively drug-resistant TB (XDR-TB) [1]. Hence, in 2019 the World Health Organization (WHO) reported that the world was not on track to reach the 2020 milestones of the End TB Strategy.

Ethiopia is one of the 14 high TB, TB/HIV and MDR-TB burden countries with incident TB, MDR/RR-TB cases and overall TB deaths of 165 000, 1600 and 26 200, respectively, in the year 2018 [1].

The most important factors for the emergence and spread of MDR-TB are mismanagement of TB treatment, inappropriate use of antimicrobial drugs or the use of ineffective formulations of drugs such as the use of a single drugs, poor quality medicines or bad storage conditions, and premature treatment interruption. Hence, bacteria can then be transmitted to other susceptible individuals.

Accumulation of point mutations in coding regions for drug targets and/or drug-converting enzymes is a major mechanism for acquiring resistance in *Mycobacterium tuberculosis* (MTB) [2]. Several studies have reported that codon 531 of the *rpoB* gene and codon 315 of the *katG* gene are found to have the highest mutational frequency for RIF resistance and INH resistance, respectively [3–6]. However, other studies conducted in Ethiopia reported that INH resistance was completely due to *katG* gene mutations without any mutations in the *inhA* gene [7,8]. There is a reported association between drug resistance mutations and specific lineages of MTB [8,9] and an association of specific mutation patterns with high patient mortality [5]. Hence, assessing the drug resistance burden and corresponding mutation patterns in MTB isolates in different countries and within regions of a country can provide better knowledge of the underlying mechanisms of drug resistance-conferring mutations. Besides serving as a basis for improving the national TB control effort to address drug-resistant TB, such studies can also provide crucial information to select the best therapeutic options and to develop novel drugs that can overcome existing resistance mechanisms. Given the significance of understanding anti-TB drug resistance mechanisms for TB control, there is a lack of adequate studies on this crucial research area in many high TB burden countries.

A few studies in Ethiopia have assessed the drug resistance burden and corresponding mutation patterns of MTB isolates detected in various regions of the country [3,8,10]. However, no study to date has assessed the frequency of anti-TB drug resistance-conferring mutations in the Tigray Region of Ethiopia. This study aimed to describe the frequency and patterns of first- and second-line drug resistance-conferring mutations of MTB isolated from pulmonary TB patients in the Tigray Region, Ethiopia.

2. Materials and methods

2.1. Study setting

This study was conducted in selected health facilities in Tigray Region, Ethiopia, including the following six hospitals: Alamata Hospital (Southern Zone); Mekelle Hospital (Mekelle Special Zone); Adigrat Hospital (Eastern Zone); Adwa Hospital (Central Zone); Shire/Suhul Hospital (North-Western Zone); and Humera/Kahsay Abera Hospital (Western Zone). Tigray Region has an estimated total population of 5.13 million [11]. The region is administratively divided into seven zones (including one special zone, Mekelle). The geographical location of the hospitals and zones of the study area are depicted in Fig. 1.

2.2. Study design and population

A hospital-based cross-sectional study design was employed. Data were collected from July 2018 to August 2019. The study population comprised all presumptive pulmonary TB cases who visited selected health facilities in the region during the study period.

2.3. Sample size and sampling procedure

The sample size was determined by taking the required minimum number of MDR-TB patients to be enrolled in the study,

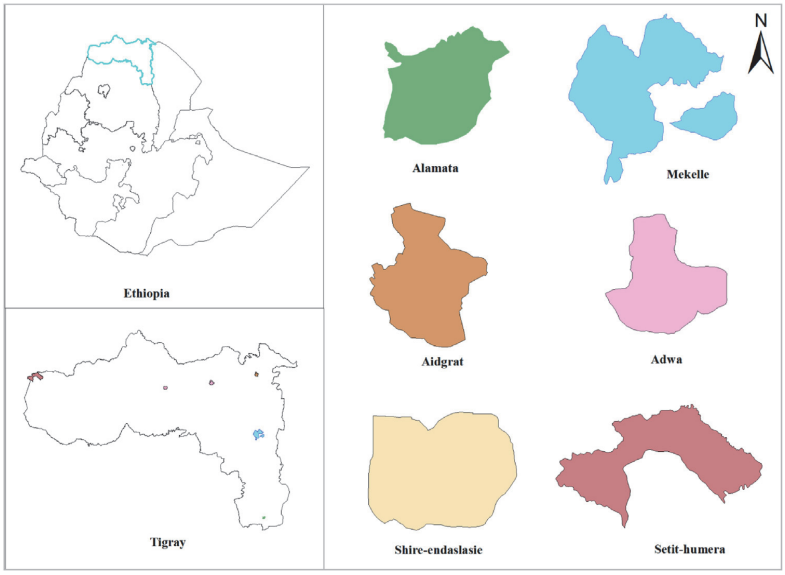

Fig. 1. Map of the study area.

which was estimated to be 30–40 cases. This was to establish a sample with a sufficient number of MDR-TB isolates to allow sufficient power to detect different types of MDR-TB isolates. Based on an expected level of 10% MDR-TB among TB patients [3], we aimed at recruiting at least 300 TB patients for the study. Without adjusting for clustering, the precision of the estimate would be 6.7–14.2% (95% confidence interval, relative precision of ±3.3%).

Hospitals from six administrative zones of Tigray Region were selected based on availability of the GeneXpert® facility. A consecutive sampling technique was employed to recruit the study sample in all of the hospitals until the required sample size was obtained.

2.4. Inclusion and exclusion criteria

Pulmonary TB patients who were not receiving treatment, aged ≥15 years with a GeneXpert®-positive result who provided written informed consent were included in the study. Critically ill patients from whom sociodemographic information, clinical data and sputum samples could not be obtained, patients aged <15 years and those with extrapulmonary TB were excluded from the study.

2.5. Sociodemographic data collection

A pre-tested structured questionnaire was employed to collect sociodemographic data, including age, sex, residence and history of previous TB treatment. Adequate training in data collection was provided to data collectors before commencement of the study.

2.6. Sputum sample collection and transportation

A 5–10 mL sputum sample was collected from eligible study participants using a coded and sterile 50-mL Falcon tube according to the recommendation of the Ethiopian Federal Ministry of Health [12]. Sputum samples were properly packed and kept at 4 °C for transportation in an ice bag to Tigray Health Research Institute (THRI) following international standards of the WHO for the transport of biological substances (category B, UN-3373). Specimens arrived within 4–5 days of collection and were processed within 7 days from the time of first collection.

2.7. Sputum decontamination procedure

Sputum samples were digested and decontaminated using freshly prepared *N*-acetyl-L-cysteine (NALC) and sodium hydroxide with a final sodium hydroxide concentration of 1%. An equal volume of NALC and sodium hydroxide solution was added to the specimen and was incubated for 15 min [13]. Following centrifugation at 3000 × *g* at 4 °C for 15 min, the sediment was re-suspended in 2 mL of sterile phosphate-buffered saline (PBS) (pH 6.8). Finally, an aliquot of 0.5 mL of sediment was inoculated into a Mycobacterium Growth Indicator Tube (MGIT) 960 and was loaded onto a BACTEC MGIT 960 instrument and two to four drops of the sediment were inoculated into Lowenstein–Jensen (LJ) egg medium. A loopful of the sediment was used for direct microscopic examination using the standard Ziehl-Neelsen staining technique at THRI [14].

2.8. Sputum culture

The decontaminated supernatant decanted sputum samples were cultured on LJ medium and in BACTEC MGIT 960 culture medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA) following standard operational procedures. Tubes for the solid culture were incubated at 37 °C in a slant position to ensure an even distribution of the inoculum for 1 week and thereafter at 37 °C in air for another 7 weeks and were checked once a week for mycobacterial growth. Cultures were considered negative when no growth was seen after 8 weeks of incubation for solid culture and 6 weeks (42 days) for liquid culture. Growth of mycobacteria was confirmed by its typical colony morphology, acid-fast bacilli staining, Capilia antigen test and inoculation onto a blood agar plate to rule out contamination.

2.9. Drug susceptibility testing for first- and second-line anti-TB drugs using the line probe assay (LPA)

All culture-positive isolates were subjected to GenoType MTBDR*plus*, and isolates that were resistant to both INH and RIF (MDR-TB) were tested again by GenoType MTBDR*sl* genotypic method following the manufacturer's instructions (GenoType® MTBC; Hain Lifescience, Nehren, Germany) [15].

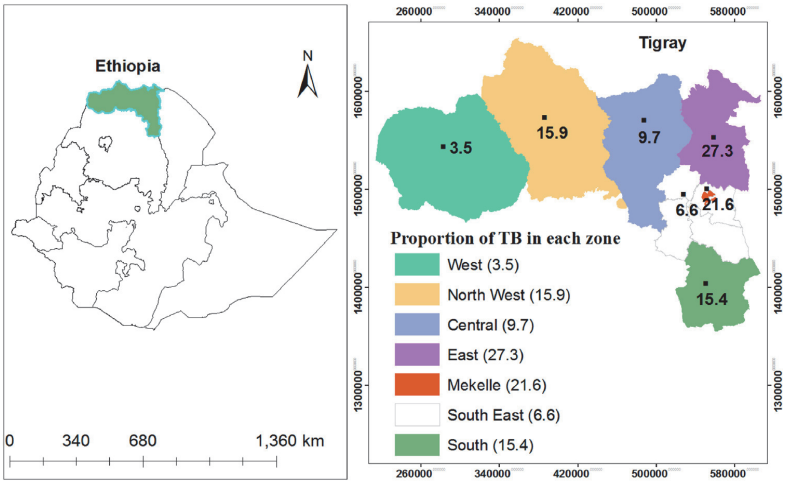

Fig. 2. Zonal distribution of the 227 tuberculosis (TB) cases in the Tigray Region, Northern Ethiopia, July 2018 to August 2019. Note: numbers are in percent.

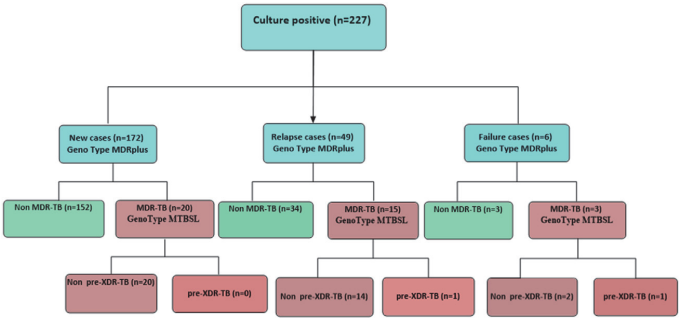

Fig. 3. Flow chart of the procedure used to recruit all participants in the study, Tigray Region, Ethiopia, July 2018 to August 2019. MDR-TB, multidrug-resistant tuberculosis; pre-XDR-TB, pre-extensively drug-resistant tuberculosis.

Table 1
Patient characteristics and frequency of resistance to first- and second-line antitubercular drugs in relation to patient tuberculosis categories, Tigray Region, Ethiopia, July 2018 to August 2019.

Variable	RIF-resistant (*n* = 40) [*n* (%)]			INH-resistant (*n* = 41) [*n* (%)]			MDR (*n* = 38) [*n* (%)]			FQ-resistant (*n* = 2) [*n* (%)]		
	New	Relapse	Failure	New	Relapse	Failure	New	Relapse	Failure	New	Relapse	Failure
Sex												
Male	9 (22.5)	13 (32.5)	2 (5.0)	11 (26.8)	12 (29.3)	2 (4.9)	8 (21.1)	12 (31.6)	2 (5.3)	0 (0)	1 (50.0)	1 (50.0)
Female	12 (30.0)	3 (7.5)	1 (2.5)	12 (29.3)	3 (7.3)	1 (2.4)	12 (31.6)	3 (7.9)	1 (2.6)	0 (0)	0 (0)	0 (0)
Age (years)												
15–24	7 (17.5)	2 (5.0)	1 (2.5)	6 (14.6)	2 (4.9)	1 (2.4)	6 (15.8)	2 (5.3)	1 (2.6)	0 (0)	0 (0)	0 (0)
25–34	6 (15.0)	7 (17.5)	1 (2.5)	7 (17.1)	7 (17.1)	1 (2.4)	6 (15.8)	7 (18.4)	1 (2.6)	0 (0)	1 (50.0)	0 (0)
35–44	6 (15.0)	4 (10.0)	1 (2.5)	7 (17.1)	3 (7.3)	1 (2.4)	6 (15.8)	3 (7.9)	1 (2.6)	0 (0)	0 (0)	1 (50.0)
45–54	1 (2.5)	1 (2.5)	0 (0)	1 (2.4)	1 (2.4)	0 (0)	1 (2.6)	1 (2.6)	0 (0)	0 (0)	0 (0)	0 (0)
≥55	1 (2.5)	2 (5.0)	0 (0)	2 (4.9)	2 (4.9)	0 (0)	1 (2.6)	2 (5.3)	0 (0)	0 (0)	0 (0)	0 (0)
Residence												
Urban	14 (35.0)	9 (22.5)	3 (7.5)	15 (36.6)	8 (19.5)	3 (7.3)	13 (34.2)	8 (21.1)	3 (7.9)	0 (0)	1 (50.0)	1 (50.0)
Rural	7 (17.5)	7 (17.5)	0 (0)	8 (19.5)	7 (17.1)	0 (0)	7 (18.4)	7 (18.4)	0 (0)	0 (0)	0 (0)	0 (0)
Total	21 (52.5)	16 (40.0)	3 (7.5)	23 (56.1)	15 (36.6)	3 (7.3)	20 (52.6)	15 (39.5)	3 (7.9)	0 (0)	1 (50.0)	1 (50.0)

RIF, rifampicin; INH, isoniazid; MDR, multidrug-resistant; FQ, fluoroquinolone.

2.10. Quality assurance and quality control

All laboratory analyses were carried out following standard operating procedures. Both the solid culture and LPA procedures were checked and validated. The MTB reference strain H37Rv was used as a quality control organism throughout the LPA test. Moreover, both start and end controls were used during each batch of specimen processing and DNA extraction, and a no-template control was also used for LPA reagents.

2.11. Data entry and statistical analysis

Data were entered using EpiData 3.1 data entry software. After cleaning and validation, data were transferred into Stata SE 15/SE for Windows (StataCorp LP, College Station, TX, USA) for statistical analysis. Descriptive statistics were computed and frequencies were presented in tables.

3. Results

A total of 227 MTB culture-positive participants were included in the study, of which 144 (63.4%) were male and 83 (36.6%) were female, with a mean ± standard deviation age of 34 ± 13.8 years (range 15–85 years). The majority of the study participants (127/227; 55.9%) were urban dwellers. The zonal distribution indicated that TB cases were highest in the Eastern Zone (27.3%), followed by Mekelle Special Zone (21.6%) (Fig. 2).

3.1. Frequency of drug resistance

Of the 227 isolates in this study, 40 (17.6%) were RIF-resistant, of which 21 (52.5%), 16 (40.0%) and 3 (7.5%) were from new, relapse and treatment failure cases, respectively. Among the 41 isolates (18.1%) resistant to INH, 23 (56.1%), 15 (36.6%) and 3 (7.3%) were detected from new, relapse and failure cases, respectively. There were two isolates (0.9%) monoresistant to RIF and three isolates (1.3%) monoresistant to INH.

The overall proportion of MDR-TB was 38 (16.7%), with the majority from new cases (20/38; 52.6%) followed by relapse cases (15/38; 39.5%) and failure cases (3/38; 7.9%). Among the 38 MDR-TB isolates tested for resistance to second-line anti-TB drugs, only 2 (5.3%) FQ-resistant isolates were detected, of which 1 was from a relapse case and 1 was from a treatment failure case (Fig. 3; Table 1).

3.2. Frequency of mutations for resistance to first- and second-line anti-TB drugs

Resistance-conferring mutations were observed in 40/227 (17.6%), 41/227 (18.1%) and 2/38 (5.3%) isolates for RIF, INH and FQs, respectively (Tables 1 and 2).

Among the 40 RIF-resistant isolates, the majority (70%) of mutations occurred at codon S531L of the *rpoB* gene, followed by mutations at codons H526Y (10%), H526D (7.5%) and D516V (5%). A total of 20% of RIF-resistant isolates were associated with the absence of wild-type (WT) probes without the development of

Table 2
Frequency of mutations conferring resistance to first- and second-line antitubercular drugs in Tigray Region, Ethiopia, July 2018 to August 2019, determined by line probe assay.

Gene	Failing WT band	Codon analysed	Developed mutation band	Amino acid change	RIF-resistant (n = 40) [n (%)]
rpoB	*rpoB* WT3	516	*rpoB* MUT1	Aspartic acid → valine	1 (2.5)
	rpoB WT3	513–517	Not detected	Unknown	2 (5.0)
	rpoB WT4	516	*rpoB* MUT1	Aspartic acid → valine	1 (2.5)
	rpoB WT4	516–519	Not detected	Unknown	1 (2.5)
	rpoB WT6	521–525	Not detected	Unknown	1 (2.5)
	rpoB WT8	531	*rpoB* MUT3	Serine → leucine	19 (47.5)
	rpoB WT8	530–533	Not detected	Unknown	2 (5.0)
	rpoB WT7	526	*rpoB* MUT2A	Histidine → tyrosine	3 (7.5)
	rpoB WT7	526	*rpoB* MUT2B	Histidine → aspartic acid	3 (7.5)
	rpoB WT7	526–529	Not detected	Unknown	2 (5.0)
	Not detected	531	*rpoB* MUT3	Serine → leucine	9 (22.5)
	Not detected	526	*rpoB* MUT2A	Histidine → tyrosine	1 (2.5)
Gene	Failing WT band	Codon analysed	Developing mutation band	Amino acid change	INH-resistant (n = 41) [n (%)]
katG	*katG* WT	315	*katG* MUT1	Serine → threonine	31 (75.6)
	katG WT	315	Not detected	Unknown	7 (17.1)
	Not detected	315	*katG* MUT1	Serine → threonine	1 (2.4)
inhA	*inhA* WT1	−15	*inhA* MUT1	Cysteine → threonine	2 (4.9)
	inhA WT2	−8	Not detected	Unknown	1 (2.4)
Gene	Failing WT band	Codon analysed	Developing mutation band	Amino acid change	FQ-resistant (n = 2) [n (%)]
gyrA	*gyrA* WT3	94	*gyrA* MUT3B	Aspartic acid → tyrosine/aspartic acid → asparagine	1 (50.0)
gyrA	Not detected	94	*gyrA* MUT3B	Aspartic acid → tyrosine/aspartic acid → asparagine	1 (50.0)
gyrA	Not detected	94	*gyrA* MUT3C	Aspartic acid → glycine	1 (50.0)
gyrA	Not detected	90	*gyrA* MUT1	Alanine → valine	1 (50.0)

WT, wild-type; MUT, mutant; RIF, rifampicin; INH, isoniazid; FQ, fluoroquinolone.

corresponding mutant bands and were reported as unknown mutations. The unknown mutations for RIF resistance occurred at *rpoB* WT3 (5%), *rpoB* WT4 (2.5%), *rpoB* WT6 (2.5%), *rpoB* WT7 (5%) and *rpoB* WT8 (5%).

Of the 41 INH-resistant strains, 32 isolates (78%) had mutations at *katG* gene codon S315T and 5% of isolates had mutations at *inhA* gene codon C15T. A total of 19.5% of INH-resistant isolates showed missing WT band without the presence of mutation bands, of which 17.1% were observed in the *katG* gene region and 2.4% in the *inhA* gene region. There was no isolate with mutations both in *katG* and *inhA* gene regions.

The most frequent resistance-conferring mutation for FQs was observed at *gyrA* codon D94Y/N (100%). However, an equal frequency of other mutations at *gyrA* codon D94G (50%) and *gyrA* codon A90 V (50%) were observed in FQ-resistant isolates (Fig. 4; Table 2)

4. Discussion

This is the first report assessing the frequency of drug resistance-conferring mutations to first- and second-line anti-TB drugs in Tigray Region, Ethiopia.

In the present study, among the 40 (17.6%) RIF-resistant isolates, 70% of the *rpoB* gene mutations occurred at codon S531L, followed by 10% at codon H526Y, 7.5% at codon H526D and 5% at codon D516V. This finding is in line with previous studies from Ethiopia as well as the findings of other studies from countries including Vietnam, Taiwan and India. At St Peter's TB Specialized Hospital in Addis Ababa (Ethiopia), the highest prevalence of mutation was observed at codon S531L (68.7%), followed by H526Y (6.6%) and H526D (4%) [16]. In Vietnam, the most common point mutations for the *rpoB* gene occurred at codon 531 (37.8%), followed by codons 526 (23%) and 516 (9.5%) [17]. In Taiwan, mutations were observed at codons 531 (49.4%), 526 (20.4%) and 516 (8.6%) [18] of the *rpoB* gene. The most frequent mutations in the study conducted in India were observed at codons S531L (57.81%), H526Y/D (14.8%) and D516V (6.42%) [19].

The frequency of mutation at codon S531L in our study is higher than in one study conducted at St Peter's TB Specialized Hospital (68.7%) [16] as well as studies in Vietnam (37.8%) [17], Taiwan (49.4%) [18] and India (57.8%) [19]. Conversely, the frequency of mutation at S531L of the *rpoB* gene observed in the current study is lower than studies conducted in another study at St Peter's TB Specialized Hospital (81.3%) [20], in Jigjiga, Ethiopia (80%) [3] and in Southwest Ethiopia (82%) [10].

Mutations in the *rpoB* gene resulting in amino acid changes within the 81-bp core region of the RNA polymerase β-subunit are found in 96% of RIF-resistant MTB strains [17]. This study revealed an overall proportion of 92.5% of mutations conferring RIF resistance, which is comparable with the findings in a report from Taiwan (91.1%) [18]. Our finding is lower than the study in Jigjiga, Ethiopia (100%) [3] and higher than the findings from St Peter's TB Specialized Hospital, Addis Ababa (82%) [16], Amhara Region (1.3%) [7], Southwest Ethiopia (85.3%) [10] and Vietnam (76%) [17].

In the current study, 20% unknown mutations conferring resistance to RIF were observed. Similarly, other studies reported that rates of unknown mutations conferring RIF resistance were 16% at St Peter's TB Specialized Hospital, Addis Ababa, Ethiopia [16], 14.7% in Southwest Ethiopia [10] and 21% in India [19]. Several studies have shown that mutations associated with RIF resistance can be found outside of the 81-bp hotspot region of the *rpoB* gene such as at codons 490, 535, 504, 541, 553 and 572 [21,22] which cannot be detected by LPA. This is supported by a DNA sequencing study performed for RIF drug resistance in Vietnam where 20.3% of strains had novel mutations at codon 490 [17]. Generally, mutations occurring in the *rpoB* hotspot region did not exhibit the same level of resistance to RIF. The most common mutations (65–86%) that occur at codons 531 and 526 are associated with a high level of resistance to RIF. Mutations at codon 516 resulted in low-level resistance of MTB to RIF. Rare mutations associated with RIF resistance have been found in the amino-terminal region of *rpoB* [21,22]. Most reference laboratories that use molecular methods (LPA) only examine the 81-bp region of the *rpoB* gene. Hence, it is recommended to screen for amino-terminal mutations to identify the presence of phenotypic RIF resistance-conferring mutations [23].

Fig. 4. Corresponding mutation patterns of rifampicin (RIF)- and isoniazid (INH)-resistant *Mycobacterium tuberculosis* (MTB) isolates by GenoType MTBDR*plus* assay. Lanes 1 and 2, mutations conferring RIF resistance at *rpoB* WT8 where the bands are absent and the corresponding *rpoB* MUT3 have appeared, and INH resistance at *katG* where the WT band is absent and the corresponding *katG* MUT1 appeared, which indicates multidrug-resistant MTB isolates. However, lanes 3–10 are susceptible isolates for RIF and INH owing to the presence of all WT bands and the absence of all MUT bands. P = H37Rv (positive control strain). N = water as a negative control. WT, wild-type; MUT, mutant.

The observed differences in the frequency of mutations could be linked to several factors. First, it may be related to the association between anti-TB drug resistance and lineage diversities of MTB strains [8]. Second, it may be due to the association of the various MTB lineages with geographic locations and the sociodemographic background of study participants [24]. A study reported the link between *rpoB* gene single nucleotide polymorphisms (SNPs) and RIF resistance, and these were MTB lineage-specific [25]. Molecular analysis of MTB strains at St Peter's TB Specialized Hospital, Addis Ababa, a national referral hospital, showed the presence of high strain diversity of MTB across the country [20].

Mutations in the catalase-peroxidase gene (*katG*) and the enoyl-acyl carrier protein reductase gene (*inhA*) have been found to account more for INH resistance [17]. Abate et al. reported that the frequency of mutations at *katG* gene codon S315T ranged from 50–95% depending on the geographic distribution [16,26], and this is in line with the current study. A low frequency of mutations at codon C15T of the *inhA* promoter (4.9%) was reported in this study. Our study finding is supported by other researchers who reported that the *katG* gene codon S315T was the most prevalent mutation responsible for INH resistance [27–29]. Besides, several study reports from Ethiopia indicated that INH resistance was due solely to *katG* mutations, and no *inhA* promoter region mutation was observed [7,8,20].

The frequency of mutations for S315T of the *katG* gene and C15T of *inhA* in the current study is higher for S315T (64%) and lower for C15T (19%) compared with a global report [30]. Moreover, the frequency of the *inhA* gene mutation in our study is lower than that reported from Southwest Ethiopia (9.8%) [10] and India (24.75%) [19], but higher than the study report from St Peter's TB Specialized Hospital, Addis Ababa, Ethiopia (0.8%) [16].

An overall mutation proportion to INH in the *katG* regulatory gene occurred in 78% of isolates. This finding is lower than the study results reported from St Peter's TB Specialized Hospital, Addis Ababa, Ethiopia (93%) [16], Jigjiga, Ethiopia (83.3%) [3], Southwest Ethiopia (90.2%) [10] and China (86.2%) [31]. The study result is higher than the findings reported from Amhara Region, Ethiopia (3.5%) [7], India (62.6%) [19] and Vietnam (76.8%) [17]. Variations in the frequency of mutations at codon S315T of *katG* and in *inhA* in the different studies largely depend on the TB prevalence in the various countries across the world and differences in geographic location [23,32]. INH remains one of the most potent first-line anti-TB drugs. The effect of this essential drug is jeopardised by the emergence of drug-resistant MTB strains. This is associated with clinically significant or high levels of INH resistance owing to the absence of significant loss of bacterial fitness resulting from frequent mutation [8,33]. Mutation at *katG* indicates a high level of resistance to INH such that treatment using INH does not have an effect on the MTB strains, whilst mutations in *inhA* indicate low-level resistance to INH and the possibility to use INH in the treatment at a high dose. Mutation at codon S315T results in an enzyme that does not have the ability to activate INH but maintains ~50% of its catalase-peroxidase activity. Thus, the altered catalase-peroxidase provides high-level resistance to INH while retaining a level of oxidative protection that is sufficient to enable the organism to maintain detoxifying activity against host antibacterial radicals [23].

The absence of any isolate with double mutations in both *katG* and the *inhA* promoter region is similar to previous studies in Ethiopia [3,10]. However, the finding is in contrast to several other study reports. Double mutations at both *katG* and *inhA* regulatory regions in the same isolate were found in studies conducted at St Peter's TB Specialized Hospital, Addis Ababa, Ethiopia (2%) [16], South India (0.1%) [28] and Shanghai, China (1.1%) [31]. These findings suggest that genetic strain background may influence the level of INH resistance conferred by particular mutations: MTB lineage 2 (Beijing genotype) was associated with any drug resistance and lineage 1 was associated with *inhA* promoter codon C15T mutations [34]. Previous studies in Ethiopia revealed that MTB lineages 1 and 2 were among the majority of lineages identified, and MTB isolates that demonstrated resistance to at least one of the tested drugs belonged to MTB lineage 2 [20,35].

Some studies indicated that most mutations linked to FQ resistance occurred in the *gyrA* gene (codons 90–94) [27,36]. Likewise, the most frequent FQ resistance-conferring mutation was observed at *gyrA* codon D94Y/N (100%) in the present study. We found an equal frequency of mutations at *gyrA* codon D94G and codon A90V. Consistent with our results, other studies showed that the most frequent mutations that confers FQ resistance were at codon 94 followed by codon 90 [36–38]. Unlike our study findings,

few other studies reported that A90V (57%) [21] and D91A (47.6%) [27] were the most frequent mutations conferring FQ resistance. Previous studies showed significant evidence regarding the association between *gyrA* mutations and FQ-resistant, MDR, pre-XDR and XDR isolates, suggesting that mutations in *gyrA* can act as a surrogate diagnostic marker for FQ-resistant and MDR-TB and a possible indicator of pre-XDR-TB or XDR-TB [27,37].

None of the isolates had mutations in the *gyrB* gene conferring resistance to FQs, concordant with study reports from Botswana [39] and Morocco [27]. Conversely, a study reported the occurrence of mutations in both *gyrA* and *gyrB* genes [37]. A systematic review by Avalos et al. and research performed in Hebei Province, China, stated that there were variabilities in the frequency of mutations in the *gyrA* gene geographically [37,40].

We were surprised to observe unexpected mutation patterns in *rpoB*, *katG* and *gyrA* genes, where both the WT and corresponding mutant band appeared. This indicates the presence of susceptible and resistant isolates, which were characterised as a hetero-resistant population of MTB strains. A high frequency of hetero-resistance was observed at *gyrA* (codons D94Y/N, D94G and A90V), followed by *rpoB* (codons S531L and H526Y) and *katG* (S315T). This finding is in line with other studies around the world [8,19,36].

This finding highlighted that there were isolates with identical mutations, which might indicate the acquisition of drug resistance that typically confers a reduction in fitness cost. This may consequently contribute to the spread of drug-resistant TB in the population. Genetic diversity analysis revealed that 28.6% of drug-resistant MTB strains were clustered, which showed the existence of clonal transmission between a small number of strains [37]. Therefore, early diagnosis and treatment with definitive anti-TB drugs and strengthening of the DOTS (directly-observed treatment, short-course) programme are imperative to prevent the transmission of acquired drug-resistant MTB in the community.

5. Conclusion

The most common resistance-conferring mutations to RIF, INH and FQs occurred at *rpoB* codon 531 (70%), *katG* codon 315 (78%) and *gyrA* codon 94 (100%), respectively. The study revealed the presence of a significant number of unknown mutations in *rpoB*, *katG* and *inhA* genes, which were associated with drug resistance to RIF and INH. Hence, these findings highlight the need for applying advanced molecular techniques (whole-genome sequencing) to help identify all genetic mutations that are relevant to drug resistance throughout the genome. Additionally, our findings suggest the need for conducting periodic surveillance of drug resistance-conferring mutations, the early diagnosis and treatment of TB, and scaling-up of drug susceptibility testing facilities to prevent and control the transmission of drug-resistant TB in the community.

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Competing interests

None declared.

Ethical approval

Ethical approval was obtained from Mekelle University, College of Health Sciences Ethical Review and Research Committee [ERC 1438/2018], the Ministry of Science and Higher Education, Ethiopia [SHE/S.M/14.4/708/19] and the Regional Committee for Medical Research Ethics in Eastern Norway (REK Øst) [2018/1118/REK sør-øst A]. Written informed consent was secured from all study participants before commencement of the study.

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PAPER III

Whole-Genome Sequencing Revealed Disputed *rpo*B mutations and Lineage-Specific Association with Drug Resistance of *Mycobacterium tuberculosis* in Tigray Region, Ethiopia

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Summary

Background

Tuberculosis, which is mainly caused by *Mycobacterium tuberculosis* (Mtb)), is an ancient human disease that gravely affects millions of people every year. This study aimed at uncovering the genetic diversity and lineage-specific association of Mtb with drug resistance among pulmonary tuberculosis patients in the Tigray Region, Ethiopia.

Methods

Sputum samples were collected from pulmonary tuberculosis patients at six different health care institutions in Tigray between July 2018 to August 2019. Deoxyribonucleic acid was extracted from 74 *Mycobacterium tuberculosis* complex (MTBC) culture-positive isolates and submitted for whole-genome sequencing (WGS) analysis. All genomes were typed and screened for mutations known to be associated with antimicrobial resistance (AMR) using *in silico* methods.

Findings

Of the 74 isolates sequenced, 68 were Mtb, three non-tuberculous mycobacteria (NTM) and three non-Mycobacteria. Lineage (L) 4 (55.8%) was predominant, followed by L3 (41.2%), L1 (1.5%) and L2 (1.5%). The frequently occurred sublineage was CAS (38.2%) followed by Ural (29.4%) and Haarlem (11.8%). The recent transmission index (RTI) was very high (80.9%). L4 was three times more resistant than L3, and Ural strains were four times more resistant than the other strains to any 1st- and 2nd-line anti-TB drugs (P <0.05). The most frequent mutations to RIF, INH, ETB, SM, PZA, ETH, FLQs and 2nd-line injectable drugs (SLIDs) occurred at *rpoB* S450L, *katG* S315T, *embB* M306I/V, *rps*L K43R, *pncA* V139A, *ethA* M1R, *gyr*A D94G and *rrs* A1401G, respectively. Besides the canonical mutations, a significant number of disputed *rpoB* mutations were also reported.

Interpretation

The WGS analysis revealed that the presence of diversified Mtb genotypes circulating in the Tigray Region, Ethiopia. The high level of RTI and presence of a significant proportion of disputed *rpoB* mutations highlighted the need to establish a WGS facility at the regional level to periodically monitor 1st- and 2nd-line DR mutations. This will contribute to better clinical management and controlling the transmission of DR-TB and for the attainment of 100% drug susceptibility test (DST) coverage for TB patients as per the End TB strategy.

Keywords: *whole-genome sequencing, disputed rpoB* mutations*, drug resistance, Mycobacterium tuberculosis, Tigray, Ethiopia*

Introduction

Tuberculosis (TB), caused by closely related species of MTBC, is an ancient human disease that continues to affect millions of people every year worldwide (1). In 2019, there were 10.0 million incident cases and approximately 1.41 million deaths due to TB (2). Ethiopia is among the top 30 high TB burden countries globally, with 157,000 incidence TB cases in 2019 (2).

Conventional genotyping, and more recently, whole-genome sequencing results indicate that MTBC is more diverse than previously anticipated globally. Different phylogenetic lineages markedly vary in their geographic distribution (3).

The seven MTBC lineages are associated with the different epidemiological profiles, host range, pathogenicity, geographic regions and drug resistance (DR) (4,5). Genomic diversity within and between MTBC lineages revealed that the most geographically widespread "generalist" L2 (Beijing), L3 (East-African-Indian) and L4 (Euro-American) are more virulent than other "specialist" lineages like L5, L6 and L7 that are more geographically restricted (4). According to the current evidence, L7 originated and remains restricted to Ethiopia, Horn of Africa (6) and has a significant evolutionary interest because of the phylogenetic positioning of Mtb L7 strains between the ancient L1 and modern lineages L2, L3 and L4 (7). A recently discovered lineages of MTBC are claimed to be restricted to the African Great Lakes region (L8) (8) and East of Africa (L9) (9).

Phylogenetic Mtb studies revealed that various genotypes entered Ethiopia as a consequence of human movement and trade. Moreover, the increased TB mortality in Africa was driven by the introduction of European strains of *Mycobacterium tuberculosis* (Mtb) together with the expansion of selected native strains having a fitness benefit in the urbanized settings of post-colonial Africa (10).

The strategies to control TB disease are rapid and accurate diagnosis and effective treatment of active cases and stopping recent transmission chains in the community (3,11). Knowledge of the transmission dynamics of Mtb is crucial from the perspective of TB management in the community (12). Whole-genome sequencing (WGS) has shown the highest resolution to accurately determine transmission dynamics and identify clusters accurately (3).

Molecular studies in the capital city (13) and the northwestern (11,12) parts of Ethiopia showed extensive strain diversity. The high proportion of new strains suggested that the genetic diversity of Mtb strains in Ethiopia has not yet been studied. Ethiopia is a demographically complex country, and it is highly unlikely that the strain composition found in the capital would be the same as in rural regions. (14). Hence, the need for further studies in the country's respective regions for proper understanding and management of TB in the country (15). No previous studies have been conducted to explore the molecular diversity and recent transmission dynamics of MTBC in the Tigray Region. Therefore, the current study aimed at assessing the genetic diversity of pulmonary TB-associated MTBC in the Tigray Region of Ethiopia using whole-genome sequencing.

Material and methods

Study area:

The study was conducted in six hospitals from the six main zones of Tigray Region, northern Ethiopia. The region has an estimated total population of 5.13 million (16) and is administratively divided into 7 Zones. All health care facilities had TB diagnostic facilities with acid-fast bacteria (AFB) smear microscopy. Moreover, some hospitals were equipped with GeneXpert instruments for rapid detection and rifampicin (RIF) sensitivity testing. The Tigray Health Research Institute (THRI) is the only facility providing TB culture and drug susceptibility testing in the region. The geographical location of the study area is illustrated in **Figure 1**.

A hospital-based cross-sectional study design was conducted from July 2018 to August 2019. Pulmonary TB patients who were not under active treatment, ≥15 years of age, GeneXpert positive and who provided written informed consent were included in the study. Critically ill patients from whom socio-demographic, clinical data and sputum samples could not be obtained, patients <15 years of age and extrapulmonary TB cases were excluded from the study.

Sputum and data Collection

Socio-demographic, clinical data, previous history of medical illnesses and behavioral factors were collected from patients using a structured questionnaire.

A 5-10 ml sputum sample was collected using coded and sterile 50 ml falcon tube from eligible study participants according to the recommendation of the Ethiopian Federal Ministry of Health (FMOH) (17). The sampled sputa were transported to THRI for AFB and culture following the WHO recommendation to transport biological substance; category B, UN-3373 (18).

The processed sputum samples were inoculated into Mycobacterium Growth Indicator Tube (MGIT 960 tube) (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and into Lowenstein-Jensen (LJ) egg media following the standard operating procedures. The direct microscopic examination using the standard Ziehl-Neelsen staining technique was done at THRI (19). Growth of mycobacteria were confirmed by its typical colony morphology, acid-fast bacilli (AFB) staining, Capilia antigen test and growth onto a blood agar plate.

Deoxyribonucleic acid (DNA) extraction and whole-genome sequencing (WGS)

DNA was extracted from 74 MTBC culture-positive isolates (26 MDR-TB and 48 mono-resistant/susceptible isolates by line probe assay (LPA)) for WGS. The susceptible isolates were

selected based on the stated criteria: duration of illness < 60 days without hemoptysis, duration of illness ≥ 60 days with hemoptysis, prison < 60 days and prison ≥ 60 days, sputum smear grade < 3 and ≥ 3, to make the isolates representative.

DNA was extracted using a combination of physical and chemical methods. In to beads containing micro-centrifuge, 400µl TE buffer (10 mM Tris–HCl, 1mM disodium EDTA (pH 8.0)) and 1 loopful colony were added. After vortexing, the solution was deactivated by heat at 94^0C for 20 minutes, then cooled down in a magnalyser cooling block (Roche) and shaken in magnalyser 3X90 second cooling down for 30 seconds in between the cycles. The suspension was removed from the beads and used as starting material for DNA isolation using EZNA Bacterial DNA Kit (Omega Bio-tek) (20). Quantitative and qualitative measurements of the DNA were performed using Qubit® 3.0 Fluorometer and NanoDrop spectrophotometers (Thermo Fisher Scientific) (21), respectively. The DNA library was prepared using a Kapa DNA Library preparation kit and sequenced by Illumina-NextSeq 500, with a Paired-End read length of 2 x 75.

Quality assurance and quality control

The quality of the WGS data was assessed with FastQC (v0.11.8) (22) and aggregated with MultiQC (v0.4) (23). This indicated a homogenous read depth (1.7 - 4.4M reads, median 3.3M) and level of read duplication (11.0 - 35.3%, median 19.0%). All isolates had mean phred quality > 30 across all read positions. No samples were found to have adapter contamination above the 0.1% level. All isolates were verified as Mtb by MASH (v2.1) (24) and verified by kraken (v2.0.7-beta) (25) using the minikraken database.

Data entry and statistical analysis

Reads were aligned to reference genome H37Rv (26) using the snippy pipeline (v4.3.6) (27). The mean read depth across the genome varied from 53.6 - 135.0, with a median of 96.8. Individual isolates were typed using the Coll *et al*. scheme (28) with the program colltyper (v0.7) (29). Microbe predictor (v0.7.0) was used to screen for mutations involved in antimicrobial resistance (AMR) (30) and independently verified with TB-profiler (31). Snippy-core (v4.3.6) was used to get whole-genome alignments of all isolates. From this, known repeat regions such as PE/PPE regions were masked, and the resultant reduced alignment was used to create a phylogenetic tree in FastTree (v2.1.10) (32) using the GTR evolutionary model.

Additionally, variant call format (VCF) files from Snippy was used to generate a visualization in Nextstrain (33). Here, repeat regions were again masked, the tree outgroup was set to isolate four, and ancestral node sequences generated at the nucleotide and protein levels for all genes. This allowed in-depth inspection of AMR emergence on branches of the phylogenetic tree. Finally, we used auspice.us to visualize these results. A cluster is two or more strains with similar genetic patterns, while nonclustered strains have different/unique genetic patterns. The recent transmission index was calculated as the number of clustered patients minus the number of clusters divided by the total number of patients (34). Univariable logistic regression was used to identify associations between lineages, sublineages and clusters with resistance to any ant-TB drugs.

Ethical considerations

Ethical clearance was obtained from Mekelle University, College of Health Sciences Ethical Review and Research Committee (ERC 1438/2018), Ministry of Science and Higher Education, Ethiopia (SHE/S.M/14.4/708/19) and Regional Committee for Medical Research Ethics in Eastern Norway (REK Øst) (2018/1118/REK sør-øst A). Written informed consent was secured from all study participants before the commencement of the study.

Results

Out of the 74 isolates subjected to WGS, 68 isolates were Mtb, three NTM (two *M. simae* and one *M. elephantis*) and three of them were non-Mycobacteria. The 68 Mtb isolates were used for further analysis in this paper. The isolates were collected from participants whose median age was 30 years, ranging from 16-66 years. Of these, 47 (69.1%) were males and 21 (30.9%) females.

The most frequent lineage was Euro-American (L4) (38; 55.8%), followed by East-African-Indian (L3) (28, 41.2%), Indo-Oceanic (L1) (1; 1.5%) and East-Asian/Beijing (L2) (1; 1.5%). The most common sublineage circulating in the region was Central Asian Strain (CAS) (26; 38.2%) followed by Ural (20; 29.4%), Haarlem (8; 11.8%), LAM (3; 4.4%), Uganda (2; 2.9%), CAS1-Kili (2; 2.9%), X-type (2; 2.9%), Cameroon (1; 1.5%), T3 (1; 1.5%), EAI2 (1; 1.5%), Beijing (1; 1.5%), TUR (1; 1.5%) and LAM7-TUR (1; 1.5%). 62 (91.2%) isolates distributed into seven distinct clusters (ranged 2-26 isolates) and six (8.8%) isolates had unique patterns, resulting in the RTI of 80.9%. Among the Pre-XDR-TB, MDR-TB and isolates resistant to any 1st- and 2nd-line anti-TB drugs, 100% (2/2), 90.9% (20/22) and 86.2% (25/29), respectively distributed within the four major clusters. Any DR to 1st- and 2nd-line anti-TB drugs were associated with L4 (OR= 2.8, 95% CI= 0.98-7.84) comparing to L3 and specifically Ural strain (OR= 3.8, 95% CI=1.11-13.46) comparing to the strains identified (**Table 1 & Figure 2**).

Geographical distribution of Mtb lineages

The Mtb lineage distribution in Tigray Regional State showed that in Alamata hospital (Southern Zone), L4 (3; 75%) was the dominant strain followed by L3 (1; 25%). Similarly, in Mekelle hospital (Mekelle Special Zone), L4 (21; 61.8%) was the dominant strain, followed by L3 (13; 38.2%) and in Adwa (central Zone), L4 (3; 100%) was the only strain isolated. L3 (9; 50%), L4 (7; 38.8%), L1 (1; 5.6%) and L2 (1; 5.6%) were found in Adigrat hospital (Eastern Zone). In Shire hospital (Northwestern Zone), L3 (4; 57%) was the dominant strain followed by L4 (3; 42.9%), and in Humera hospital (Western Zone), equal distribution of L3 (1; 50%) and L4 (1; 50%) were observed (**Table 2 & Figure 3**).

Genetic determinants of DR-TB

Among the 68 MTBC isolates, 22 (32.4%) were MDR-TB, two (2.9%) pre-XDR, five (7.4%) resistant to one or more anti-TB drugs and 39 (57.4%) were pan-susceptible. Of the 25 isolates resistant to RIF, the predominant mutations occurred at *rpoB* codon S450L (18; 72%), followed

by H445N (2; 8%). Mutations at *rpoB* codons H445Y, H445D, D435Y, D435V and L430P occurred at one (1; 4%) isolate for each. Except for one isolate which showed mutation at *fab*G1gene codon C-15T (1; 3.8%), the 25 isoniazid (INH) resistant isolates showed mutation at *katG* gene codon S315T (25; 96.2%).

The main resistance conferring mutations for the 18 isolates resistant to Ethambutol (EMB) occurred at *embB* gene codon M306I (4; 22%), D328G/Y (4; 22.2%), G406A (3; 16.7%), M306V (2; 11%), Q497K (1; 5.6%) and D354A (1; 5.6%). Double mutations were detected at *embB* gene codons M306I and G406A (1; 5.6%), G406A and D1024N (1; 5.6%), and M306I and G406S (1; 5.6%).

The most prevalent mutation for streptomycin (SM) resistance isolates (24 isolates) was detected at *rps*L gene codon K43R (14; 58%). Three (12.5%) isolates showed double mutation at both *rpsL* gene codon K88T and *gid* gene codon G69D. Additionally, SM resistance-conferring mutations were observed at *rps*L gene codon K88T, *rrs* gene codons A514C and C517T, *gid* gene codons 115del, 351del, 102del and G69D from one (4.2%) isolate each.

Resistance conferring mutations to pyrazinamide (PZA) resistant isolates (10 isolates) occurred at *pncA* gene codons V139A (2; 20%) and F58L, F13L, G46G, P69L, T76P, V180F, Q141P and D49A in one isolate each (10%). One isolate (10%) showed a silent mutation at *pncA* gene codon G46G. Among the 11 isolates resistant to Ethionamide (ETH), five (45.5%) isolates showed resistance-conferring mutations at *ethA* gene codon M1R, one (9%) at *fab*G1 gene codon C-15T and five (45.5%) isolates showed mutations at other codons (1407del and 1341del).

The DR conferring mutation for 2nd-line anti-TB drugs occurred at *gyr*A codon D94G for FLQs resistance and *rrs* gene codon A1401G for SLIDs (AMK, KAM and CAP) in one isolate for each. Additionally, mutation at *tly*A gene codon N236K was detected in another one isolate resistant to CAP (**Table 3**).

Discussion

WGS uncovered the diversified lineage makeup and its distribution in the Tigray Region, Northern Ethiopia. The geographic distribution of lineages showed that L4 was dominantly isolated from Alamata hospital, Mekelle hospital, and in Adwa hospital, the only lineage isolated. In contrast, L3 was dominant in Adigrat hospital and Shire hospital. However, in Humera hospital, equal distributions of L3 and L4 were observed. Surprisingly, L1 and L2 were isolated only in Adigrat hospital. This difference could be due to the patient location difference (35).

In the present study, the predominant lineage was L4, followed by L3. This is in agreement with a study report from St. Peter's TB Specialized Hospital, Ethiopia (13), Amhara Region, Northwest Ethiopia (14,36), Southern Ethiopia (37), Central Ethiopia (38), and African countries like Sudan (39) and Botswana (40). Similarly, a nationwide review and community-based survey in Ethiopia showed that the most frequently isolated lineage was L4, followed by L3 and L1 (41,42). L4 isolates accounted for 91% of TB cases in Europe and the Americas, the Caribbean, the Middle East, and all Africa regions.

In the current study, EAI2/L1 was recovered from a single patient. Other study reports from Ethiopia (37,38), Sudan (39), Tanzania (43) and Botswana (40) reported a higher proportion of L1. Likewise, a single isolate of L2/Beijing was observed in our study which is in line with former studies conducted in most parts of Ethiopia (14,15,44,45). In contrast, L2/Beijing was not isolated in most Ethiopia parts (13,37,46). However, L2/Beijing has been isolated in higher proportions in other African countries (40,43,47–49). The isolation of the Beijing strain associated with MDR-TB indicates its recent transmission into a geographic area through the migration of people from East Asia (50–52). In Ethiopia, the isolation of L2/Beijing was reported in 2013 (14). Since then though in low frequency, its isolation in different parts of Ethiopia is alarming for the increasing dissemination of DR-Beijing strain throughout the country (15,44,45)

Surprisingly, L7 was not reported in the present study, while it is prevalent in the Amhara Region, Northwestern Ethiopia (7,42) and less frequently in South Omo, Southern Ethiopia (37). Likewise, L7 was not reported in the Afar Region, Northeast Ethiopia (46), Central Ethiopia (38) and Sudan (39), bordering both Tigray and Amhara Regions, indicating its geographical restriction mainly to the Amhara Region, Ethiopia.

A nationwide review report from Ethiopia showed that L1 was relatively more common in Afar Region, L3 and L7 in Amhara Region, and L4 in Oromia Region, Southern Nations Nationalities and Peoples, Central Ethiopia and Southeastern Ethiopia (42). L1 is commonly associated with populations living around the Indian Ocean, L3 is common in Central Asia and prevalent in East Africa, and L4 is common in European-American (10). The variation in the distribution of lineage could be associated with variation in the geographic region, ethnic group, age and sex of patients (52).

According to the WGS analysis, in the Tigray Region, the dominant sublineage was the CAS strain. This finding is consistent with previous studies from Southern Ethiopia (37), Northwest Ethiopia (15), and African countries like Tanzania (43). However, other studies from Southwest Ethiopia (53) and Northwest Ethiopia (14) reported the T (Tuscany) family. The National Tuberculosis Reference Laboratory, Addis Ababa, Ethiopia identified T3_ETH (54). Studies in Kenya and Sudan reported CAS1-KILI (49) and Manu2 (39) as the dominant strains. The CAS sublineage is dominant in the Indian subcontinent, South-East Asia, the Middle-East and East-Africa (46). These molecular studies revealed that the presence of diversified strains and the transmission pattern of Mtb is different across the world.

The second-largest sublineage in this study was Ural, followed by Haarlem, LAM, Uganda, CAS1-kili, X-type, LAM7-TUR, Cameroon, T3, EAI2, Beijing and TUR. The sublineages like Cameroon, Uganda and CAS1-kili were not reported in various parts of Ethiopia (37,53,55,56). In contrast, lineages like *M. africanum* (38,46) and *M. bovis* (36,46,55), and sublineages like CAS1-Delhi and Manu1/2 which were reported in different parts of Ethiopia (36,38,53,55), were not reported in our study. In Ethiopia, the major lineage and sublineages are highly diversified within the country. The composition and proportion of these genetic diversities varied with time. Previous reports revealed that L4 sublineage H is slightly higher in the Amhara Region, S is more frequent in Oromia; however, T is relatively lower in Benishangul Gumuz (42).

Moreover, the country mapping each sublineage's proportion indicated that the distribution greatly differed by geographic location. Specifically, L4 sublineages Haarlem, LAM and L4.10/PGG3 are distributed across the world and are called generalists, while specialist sublineages Ghana, Uganda and Cameroon occurred at high frequencies but in specific regions of Africa or Asia but were

almost absent from Europe and the Americas. The X sublineage mainly occurred in the Americas and at lower proportions in Southern Africa, Asia and Europe. L4.1/X, L4.2/Ural and L4.4 occurred in high proportions in Asia and Africa but were mostly absent from the Americas are called intermediate (57). The possible reason for the various geographic distribution of generalist and specialist sublineages could be intrinsic biological factors, extrinsic factors such as human migration, or both (57). Though some genotypes already existed in the African continent before the European contact, various genotypes were introduced to Ethiopia through human migration and trade (10).

Generally, in Africa, Mtb strains are more diversified geographically. Both generalist and specialist genotypes are circulating in the region. L4 is the dominant lineage across the continent, whereas *M. bovis* is rarely isolated from TB patients. TB transmission in Africa is characterized by both clustering and reactivation of Mtb strains (58).

Clustering reflecting recent and active transmission of TB depends on study duration (59). According to the WGS analysis, the overall clustering and RTI was 91.2% and 80.9%, respectively. This finding is higher than the overall clustering rate and RTI of previous studies in other regions of Ethiopia: Southern Ethiopia 57.7% and 3.9% (37), Northwest Ethiopia 30.3% and 45.1% (15), Northeastern Ethiopia 76.2% (46), central Ethiopia 79.8% and 68% (38), and a nationwide review in Ethiopia 41% and 29% (42), respectively. Besides these, our finding is higher than the clustering rate reported from Morocco (60), Botswana (40) and South Africa (48), but was more or less similar to Russia (79.3%) (61). Clustering indicates ongoing transmission in the community and the unique pattern indicates reactivation or recent introduction of Mtb strains into the geographic area. The high RTI and clustering rate in the study area shows the high transmission of Mtb strains with a low rate of reactivation or introduction of new strains. The present finding agrees with the previous study report that showed a high proportion of MDR-TB among new pulmonary tuberculosis (PTB) cases in the study area (62). The possible reason for the variation of TB transmission status could be the difference in socio-demographic status, study population and the strains (63). The other reason could be method difference; most of the studies used Spoligotyping and MIRU-VNTR, which have lower power than WGS for transmission analysis (14,42).

Study reports showed the association of the genetic background of Mtb strain and drug resistance (64). Our finding revealed that 100% of Pre-XDR-TB, 90.9% MDR-TB and 86.2% of the isolates resistant to any 1st- and 2nd-line anti-TB drugs distributed within the four major clusters (CAS, Ural, Haarlem and LAM). L4 (OR= 2.8, 95% CI= 0.98-7.84) was three times more DR than L3, and particularly sublineage Ural (OR= 3.8, 95% CI=1.11-13.46) was four times resistant to any anti-TB drugs than the other sublineages circulating in the region. A study report from Northwest Ethiopia indicated that Haarlem's sublineage was associated with resistance to 1st-line anti-TB drugs and MDR (15). Earlier studies reported the association of W-Beijing with EMB and SM-DR (61), Beijing strains with MDR-TB (52) and L2 with any DR (64). This variation could be due to the difference in the dominance of strains in the respected geographic area. The different DR-Mtb lineages were associated with different geographic areas (52). L4 was the dominant lineage in our study area, and the Ural is the second-largest sublineage isolated.

DST using the WHO recommended LPA assay detected only RIF and INH, FLQs and SLIDs resistance in the common resistance-conferring regions (65). WGS enables rapid detection of drug resistance to all 1st-and 2nd-line anti-TB drugs throughout the genome simultaneously (66), which could provide more information for clinical treatment, especially MDR-TB (66). Moreover, WGS is a promising approach to predict resistance to INH, RIF, PZA, LFX, SM and SLIDs with satisfactory accuracy, sensitivity, and specificity of over 85% (66).

In the current study, a significant proportion of disputed mutations (16%) that cause RIF resistance was observed at *rpoB* codons H445N (526), D435Y (516) and L430P (511), which were not detected in our previous study using the WHO recommended LPA analysis. The 20% of RIF resistance-conferring mutations were unknown using LPA (67). The occurrence of disputed *rpoB* mutations is clinically and epidemiologically highly relevant (68). According to the WGS analysis, 96.2% of mutations that conferred INH resistance occurred at *katG* codon S315T, which differed from our previous study that reported a 78% mutation at *katG* codon S315T and 19.5% unknown mutation using LPA (67).

Mutations at *emb*B, *emb*C and *emb*A genes are responsible for EMB-R (69). However, in this study, mutations were detected at *emb*B gene only with the most prevalent codon M306I (22.1%) followed by G406A (16.7%), D328G/Y (22.2%), M306V (11%), Q497K (5.6%), and D354A

(5.6%). Three isolates showed double mutations at *embB* gene codons M306I and G406A (5.6%), G406A and D1024N (5.6%), and M306I and G406S (5.6%). The study report showed that most mutations that cause EMB-R occurred at the *emb*B gene, particularly associated with codon 306 which enlightens its use as a surrogate marker for rapid detection of EMB-R (70–73). However, mutation at *emb*B306 is also detected in susceptible isolates but is significantly lower than in the resistant isolates (73).

Mutations that arise SM-R are linked to genes *rrs*, *rps*L, and *gid*B, encoding 16S rRNA, ribosomal protein S12, and 16S rRNA specific methyltransferase, respectively (74). According to our finding, SM-R's most prevalent mutation was detected at *rps*L gene codon K43R (58%). Three (12.5%) isolates showed co-existed mutations at both *rpsL* gene codon K88T and *gid* gene codon G69D. Additionally, SM-R conferring mutations were observed at *rps*L gene codon K88T, *rrs* gene codons A514C and C517T, *gid* gene codons 115del, 351del, 102del and G69D in one (4.2%) isolate each. Though the proportion of mutation at K43R varied geographically, this finding is concordant with an earlier study that reported its dominance across the world and its association with a high DR level (74,75). Mutations that occurred at either *rrs* or *rps*L genes were the main mechanism and entirely linked with SM-R; hence they could serve as molecular markers for detecting SM-R (74). The mutations detected at *gid*B were detected at both susceptible and resistant isolates even though certain point mutations were associated with DR in LAM and Beijing genotypes, indicating its phylogenetic usefulness markers rather than SM-R markers (74,75).

The current WGS analysis revealed that the resistance-conferring mutations to PZA occurred at *pncA* gene codons V139A (20%) and F58L, F13L, G46G, P69L, G46G, T76P, V180F, Q141P and D49A in one isolate each (10%). The mutations seem to be concentrated at codons 46-76 and 139-141. Though mutation that occurred at *pncA* gene is the major mechanism of PZA-R, amino acid substitution varies among studies. The most common mutation that ensues PZA-R was detected at *pncA* gene (codons 3–17, 61–85, and 132–142) in PZase active and metal-binding sites (76), and other report indicated that the mutations were dispersed along the *pncA* gene with some clustering at *pncA* gene codons 132-142, 69-85, and 5-12 (77).

ETH is a structural analogue of INH, which both of the drugs targeted at *Inh*A enzyme responsible for mycolic acid biosynthesis (78). ETH conferring mutations occurred at *ethA* gene codon M1R

(45.5%), *fab*G1 gene codon C-15T (9%) and five (45.5%) isolates at another codon (1407del and 1341del). Our finding revealed that all isolates resistant to ETH were co-resistant to INH, which corresponds for those that had mutations at *ethA* gene codon M1R and other codons (1407del and 1341del) were also mutated at *kat*G gene codon S315T. One isolate had *fab*G1 gene codon C-15T mutation reported at both ETH and INH. This finding is supported by prior study reports that showed the isolation of Mtb strains co-resistant to INH and ETH from TB patients previously treated with INH but never treated with ETH (79). The *fab*G1 gene codon C-15T mutation, which conferred resistance to both INH and ETH, was detected from MDR-TB. This is consistent with another study report that revealed mutation at *inh*A promoter region codon c-15t is detected in a large proportion of resistant isolates to INH and ETH. Its occurrence is in high proportion among XDR-TB cases, suggesting that this mutation could be a marker for XDR-TB (79).

In our previous study report, drug resistance-conferring mutations to 2nd-line anti-TB drugs developed at *gyr*A gene codon D94G, D94Y/N and A90V, but mutations that confer resistance to SLIDs were not reported (67). However, the present study showed mutations at *gyr*A gene codon D94G and mutations to SLIDs.

Mutation of *rrs* gene that encodes 16S rRNA bacterial subunit is associated with high-level DR to KAN, AMK and rarely to CAP located between nucleotides 1400 and 1500 (80). According to the WGS analysis, the mutation that conferred drug resistance to AMK, KAM and CAP was detected at *rrs* gene codon A1401G from one isolate each. Another mutation occurred at *tly*A codon N236K in one isolate that confers resistance to CAP. This agrees with the previous study report, which suggests could be used as a surrogate marker for the high-level resistance to KAN and AMK (81). A study revealed that mutation at *tly*A codon N236K confer resistance to CAP was detected in many Cameron sublineages which were suggested as a phylogenetic marker for Cameroon sublineages (82). However, in our study, this mutation is detected in Uganda's sub-lineage.

In conclusion, the WGS analysis revealed that the presence of diversified Mtb genotypes circulating in the Tigray Region, Ethiopia. Overall, L4 was the most frequently observed Mtb genotype comprising the highest proportion of drug resistance in the study region. The high level of RTI indicates the rapid dissemination of DR-TB in the region. The study highlighted the usefulness of mutations at *rpoB*, *katG*, *embB*, *rps*L, *pncA*, *ethA*, *gyr*A and *rrs* genes as a molecular

marker for the rapid detection of resistance to RIF, INH, ETB, SM, PZA, ETH, FLQs and SLIDs, respectively. Given the observed high burden MDR-TB and a significant proportion of disputed *rpoB* mutation, there is an urgent need to scale up rapid testing and detection of MDR/RR-TB cases in the region and consider establishing a WGS facility at the regional level so that 1st- and 2nd-line drug-resistant mutations can periodically be monitored. This will help control the transmission of DR-TB and ultimately contribute to the attainment of 100% DST coverage for TB patients as per the End TB strategy.

Research in context

Evidence before this study

Accurate DST of Mtb to 1st- and 2nd- line drugs is crucial for early and proper management of MDR-TB patients. Mtb strains' resistance to RIF is mainly due to the canonical mutations in the hot-spot region of the *rpo*B gene (HSRrpoB). However, there are also disputed *rpoB* mutations that confer RIF-R, which are considered to occur rarely. We searched the PubMed database for articles published in all languages before February 2021, using the words "*mycobacterium tuberculosis*", "Whole-genome sequencing", "disputed *rpo*B mutation" and "drug resistance". Previous study reports by Rigouts *et al.* in 2013 and VAN DEUN *et al*. in 2009 indicated that the low-level but probably clinically relevant mutations at *rpoB* gene, mainly L511P, D516Y, H526N, H526L, H526S, and L533P in HSR-*rpo*B gene called "disputed *rpoB mutations*" are missed by the standard phenotypic DST. According to this finding, though these mutations' overall impact depends on the frequency of their occurrence, which may vary geographically, these mutations are associated with poor clinical outcomes to 1st-line treatment. Abanda et al., in 2019 reported that the WHO-recommended molecular techniques like LPA could not detect the specific disputed *rpoB* mutation. However, the missing wild-type probe without any hybridization of the mutation probe can be considered as disputed *rpoB* mutation and reported as RIF-R. In 2018, Jeong and colleagues' case report showed that patients with disputed rpoB mutation had shown successful outcomes with a high-dose (20 mg/kg) RIF-based regimen. Ahmad and Makaddas in 2014 reported treatment of MDR-TB is more expensive, more toxic, and requires prolonged treatment time, which can result in clinical failure or disease relapse than the drug-susceptible TB. So far, few countries like the Democratic Republic of Congo, Bangladesh, South Korea, China, Kuwait and Belgian reported disputed *rpoB* mutations with significant variation in the proportion. In sub-Saharan Africa, very limited studies are available reporting the occurrences of disputed *rpoB* mutations by utilizing whole-genome sequencing analysis.

Added-value of this study

The study showed the presence of a high proportion of disputed *rpoB* mutation. Our study's information enlightened the usefulness of WGS on simultaneous DST to all anti-TB drugs to halt transmission of DR-TB and ultimately contribute to the attainment of 100% DST coverage for TB patients as per the End TB strategy. Early and proper drug susceptibility test is crucial for the

proper management of patients and control of MDR-TB. The high proportion of Mtb strains with disputed *rpoB* mutations in the study area highlighted the need for the WGS to better patient management.

Implications of all the available evidence

The WGS analysis provides information about the diversity of lineages, RTI and drug resistance patterns simultaneously. The WHO recommended molecular techniques for DST like the gene Xpert and LPA could not detect resistance to all anti-TB drugs. Though the proportion varied geographically, the occurrence of disputed *rpo*B mutations of the RIF-R Mtb strains are clinically and epidemiologically highly relevant. The findings can guide clinicians in the management of patients carrying isolates with disputed *rpoB* mutations.

Consent to publish
Not applicable

Availability of data and materials
The data of this study will be available upon acceptance.

Competing interest
None of the authors had a competing interest.

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Contributors
LN, SA, ES and TA conceived and designed the study. LN collected the data and drafted the manuscript. LN and HH investigated the laboratory work. OB and LN contributed formal analysis of the data. LN, ES, SA, TA, TT, HH and OB contributed to reviewing and editing the manuscript. ES, SA and TA jointly supervised the study. All authors have seen and approved the final report.

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Table 1: Frequency and DR association with the Mtb strains and clustering status

Variable	N (%)	Pan susceptible, F (%)	Any DR, F (%)	COR (95% Cl)	P-value
Lineage					
L3	28 (41.1)	20 (71.4)	8 (28.6)	1	
L4	38 (55.9)	18 (47.4)	20 (52.6)	2.8 (0.98-7.84)	**0.054**
Sublineage					
CAS	26 (38.2)	18 (69.2)	8 (30.8)	1	
Ural	19 (27.9)	7 (36.8)	12 (63.2)	3.86 (1.11-13.46)	**0.034**
Haarlem	8 (11.8)	3 (37.5)	5 (62.5)	3.75 (0.72-19.64)	0.118
LAM	3 (4.4)	3 (100)	-	-	-
Uganda	2 (2.9)	1 (50)	1 (50)	2.25 (0.12-40.65)	0.58
CAS1-kili	2 (2.9)	2 (100)	-	-	-
X-type	2 (2.9)	2 (100)	-	-	-
LAM7-TUR	1 (1.5)	-	1 (100)	-	
Cameroon	1 (1.5)	1 (100)	-	-	-
L4.6.2/T3	1 (1.5)	-	1 (100)	-	-
EAI2	1 (1.5)	1 (100)	-	-	-
Beijing	1 (1.5)	-	1 (100)	-	-
TUR	1 (1.5)	1 (100)	-	-	-
Clustering					
No	6 (8.8)	3 (50)	3 (50)	1	
Yes	62 (91.2)	36 (58.1)	26 (41.9)	0.72 (0.13-3.87)	0.7

Note: N= number, F= frequency, COR= crud odds ratio, DR= drug resistance

Table 2. Distribution of Mtb strains isolated from PTB patients, Tigray Region Ethiopia, July 2018 to August 2019

Study site (hospitals)	N (%)	DR profile	L1, F (%)	L2, F (%)	L3, F (%)	L4, F (%)	Total, F (%)
		Pre-XDR	-	-	-	-	-
Alamata	4 (5.9)	MDR	-	-	-	2 (50)	2 (50)
		DR				1 (25)	1 (25)
		Pan susceptible	-	-	1 (25)	-	1 (25)
		Pre-XDR	-	-	1 (2.9)	1 (2.9)	2 (5.9)
Mekelle	34 (50)	MDR	-	-	3 (8.8)	10 (29.4)	13 (38.3)
		DR			1 (2.9)	-	1 (2.9)
		Pan susceptible	-	-	8 (23.5)	10 (29.4)	1 (52.9)
		Pre-XDR	-	-	-	-	-
Adigrat	18	MDR	-	-	1 (5.6)	1 (5.6)	2 (11.1)
	(26.5)	DR		1 (5.6)		1 (5.6)	2 (11.1)
		Pan susceptible	1 (5.6)	-	8 (44.4)	5 (27.8)	14 (77.8)
		Pre-XDR	-	-	-	-	-
Adwa	3 (4.4)	MDR	-	-	-	1 (33.3)	1 (33.3)
		DR				2 (66.7)	2 (66.7)
		Pan susceptible	-	-	-	-	-
		Pre-XDR	-	-	-	-	-
Shire	7 (10.3)	MDR	-	-	1 (14.3)	1 (14.3)	2 (28.6)
		DR				1 (14.3)	1 (14.3)
		Pan susceptible	-	-	3 (42.9)	1 (14.3)	4 (57.1)
		Pre-XDR	-	-	-	-	-
Humera	2 (2.9)	MDR	-	-	1 (50)	1 (50)	2 (100)
		DR	-	-	-	-	-
		Pan susceptible	-	-	-	-	-
Total	68 (100)		1 (1.5)	1 (1.5)	28 (41.2)	38 (55.8)	68 (100)

Note: N= number, F= frequency, DR= drug resistance, MDR= multi drug resistant, Pre-XDR= Pre-extensively drug resistant

Table 3: Frequency of gene mutation for 1st-and 2nd-line anti-TB drugs

Drug	Target gene	Mutation	N (%) ≥1 DR	N (%) MDR-TB	Pre-XDR	Total (N)
		S450L	-	16 (64)	2 (8)	18 (72)
		H445N	-	2 (8)	-	2 (8)
		H445Y	-	1 (4)	-	1 (4)
RIF	*rpoB*	H445D	-	1 (4)	-	1 (4)
(n=25)		D435Y	-	1 (4)	-	1 (4)
		D435V	-	1 (4)	-	1 (4)
		L430P	1 (4)	-	-	1 (4)
INH (26)	*katG*	S315T	2 (7.7)	21 (80.8)	2(7.7)	25 (96.2)
	fabG1	C-15T	-	1 (3.8)	-	1 (3.8)
		M306I	1(5.6)	3 (16.7)	-	4 (22.1)
		D328G/Y		4 (16.7)	-	4 (22.2)
		G406A	-	3 (16.7)		3 (16.7)
EMB (18)		M306V	-	2 (11.1)	-	2 (11)
	embB	Q497K	-	1 (5.6)	-	1 (5.6)
		D354A	-	-	1(5.6)	1 (5.6)
		M306I; G406A	-	-	1(5.6)	1 (5.6)
		G406A; D1024N	-	1 (5.6)	-	1 (5.6)
		M306I; G406S	-	1 (5.6)	-	1 (5.6)
	gid	115del	1 (4.2)	-	-	1 (4.2)
		351del	-	1 (4.2)	-	1 (4.2)
		102del	-	1 (4.2)	-	1 (4.2)
		G69D	-	1 (4.2)	-	1 (4.2)
SM (24)	*rpsL, gid*	K88T; G69D	-	3 (12.5)	-	3 (12.5)
	rpsL	K88T	-	1 (4.2)	-	1 (4.2)

		K43R	1 (4.2)	12 (50)	1 (4.2)	14 (58)
	rrs	A514C	-	1 (4.2)	-	1 (4.2)
		C517T	-	-	1 (4.2)	1 (4.2)
		V139A	-	2 (20)	-	2 (20)
		F58L	-	1 (10)	-	1 (10)
		F13L	-	1 (10)	-	1 (10)
		P69L	-	1 (10)	-	1 (10)
		G46G	-	1 (10)	-	1 (10)
PZA (10)	*pncA*	T76P	-	1 (10)	-	1 (10)
		V180F	-	1 (10)	-	1 (10)
		Q141P	-	1 (10)	-	1 (10)
		D49A	-	1 (10)	-	1 (10)
ETH (11)	*ethA*	M1R	-	5 (45.5)	-	5 (45.5)
		other	-	4 (36.4)	1 (9)	5 (45.5)
	*fab*G1	C-15T	-	1 (9)	-	1 (9)
FLQs (1)	*gyr*A	D94G	-	-	1 (100)	1 (100)
AMK (1)	*rrs*	A1401G	-	-	1 (100)	1 (100)
KAM (1)	*rrs*	A1401G	-	-	1 (100)	1 (100)
CAP (2)	*rrs*	A1401G	-	-	1 (50)	1 (50)
	tlyA	N236K	1 (50)	-	-	1 (50)

Note: N= number, DR= drug resistance, MDR= multi drug resistant, Pre-XDR= Pre-extensively drug resistant

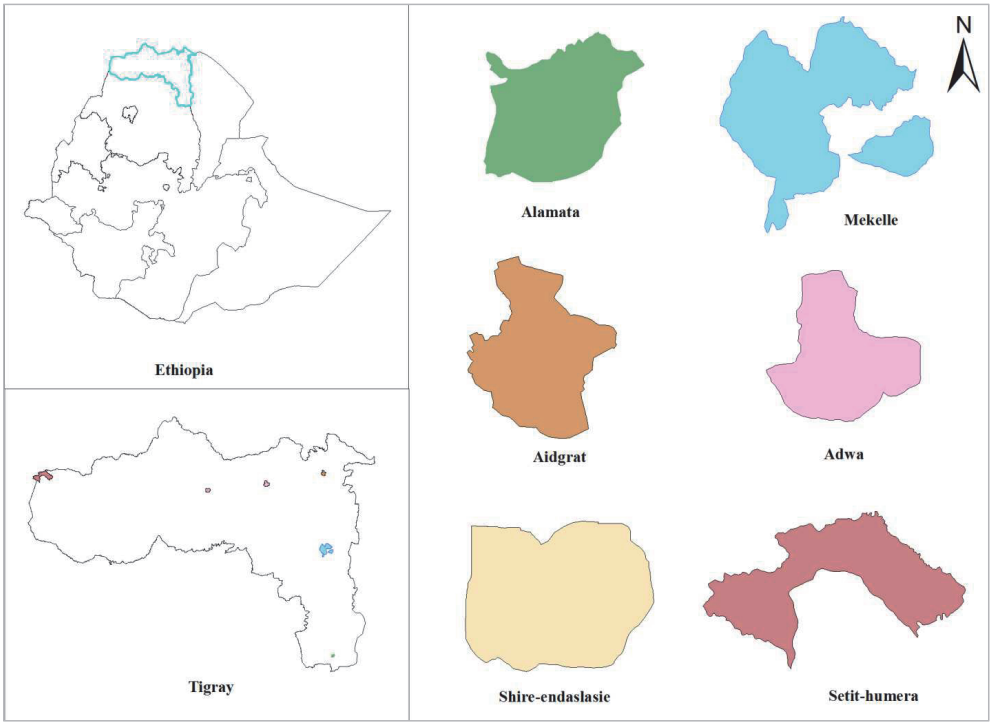

Figure 1: Map of the study area showing locations of hospitals

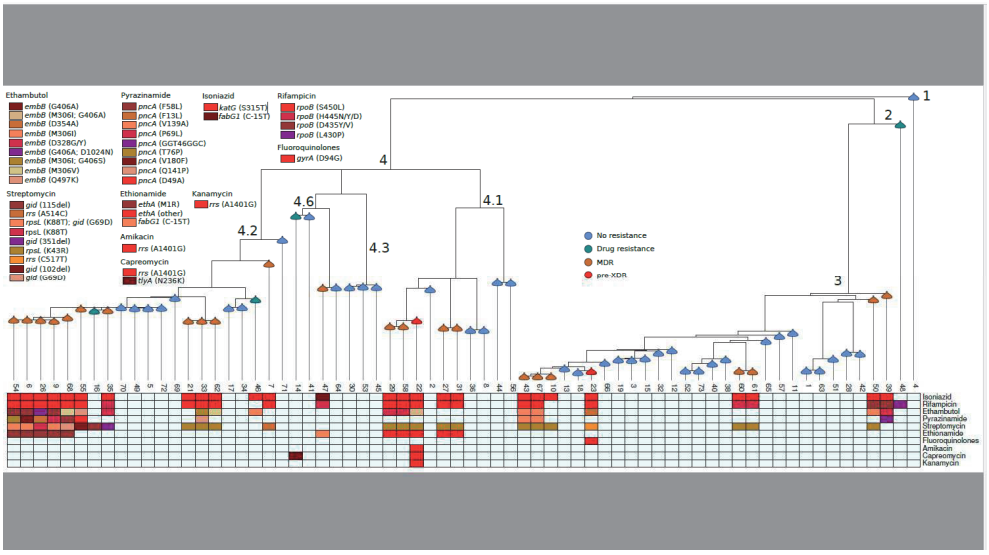

Figure 2: MTBC included in this study. Branches are annotated with lineage and nodes are annotated with drug resistance pattern. Boxes at the bottom part of the figure display specific AMR-associated mutations.

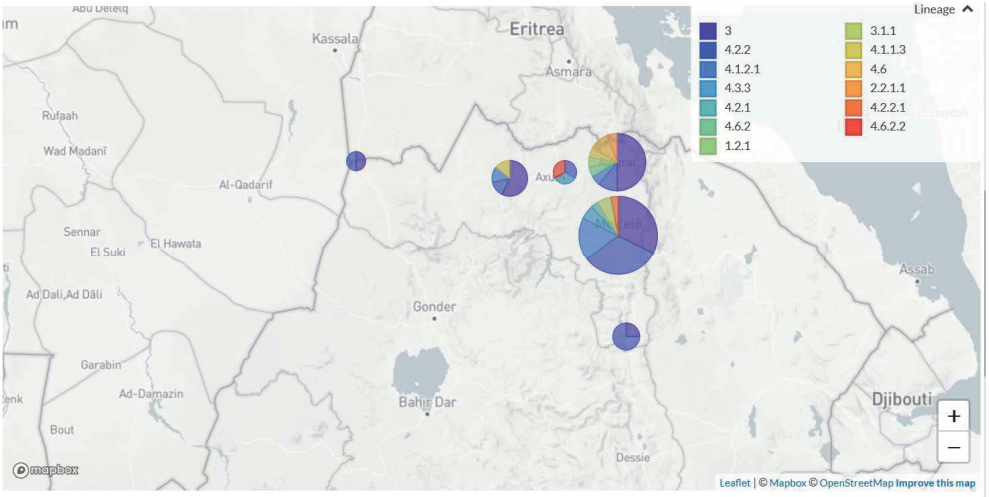

Figure 3: The distribution of lineages in the Tigray Region, Ethiopia

Appendix II: Additional files

Questionnaire: https://doi.org/10.1371/journal.pone.0236362.s002

INFORMED CONSENT FORM (≥18 years old)

By signing below, I confirm the following:

- I have been given oral and written information about the proposed study; have read/been read and understood the information given
- I have had sufficient time to consider participation in the study and have had the opportunity to ask questions and all my questions have been answered satisfactorily
- I know that participation in this project is voluntary, and I have understood the content and the objective of the study
- I am further aware that the information and specimen I will provide will only be utilized in the proposed study
- I recognize that all the information regarding myself will be kept completely confidential, and I know that I can quit any time from this study without providing any reason for doing so
- I know that sputum or culture (*M. tuberculosis*) will be transferred to Norway, veterinary institute for advanced laboratory analyses.

It is therefore with full understanding of the situation that I am giving my voluntary informed consent

Name_____Signature_____Place_____Date_____

Witnesses name (for those who do not read and write)

1. Name _____Signature _____

Name of the person who take consent _____

signature_____Place_____Date_____

INFORMED ASSENT FORM (15-17 Years of Age)

By signing below, I confirm the following:

- I have been given oral and written information about the proposed study; have read/been read and understood the information given.
- I have had sufficient time to consider participation of my child/relative in the study and have had the opportunity to ask questions and all my questions have been answered satisfactorily.
- I know that participation in this project is voluntary, and I have understood the content and the objective of the study.
- I am further aware that the information and specimen my child /relative will provide will only be utilized in the proposed study
- I recognize that all the information regarding my child/relative will be kept completely confidential, and I know that I can quit any time from this study without providing any reason for doing so.
- I know that sputum or culture (M. *tuberculosis*) will be transferred to Norway, Veterinary institute for advanced laboratory analyses.

It is therefore with full understanding of the situation that I am giving my voluntary informed consent

Name_____Signature_____Place_____Date____

Name of family/next of kin_____ Signature_____ Place_____ Date____

Witnesses name (for those who do not read and write)

1. Name _____Signature _____

Name of the person who take consent _____

signature_____Place_____Date_____

Questionnaire for patients

Date of data collection:_____E.C

Name of data collector and signature:_____Mobile:_____

Name of Health institution (Health center /Hospital): _____Zone:_____

Patient Card number:_____sputum Code (assigned by data collector):_____

Gen X-pert Results

A. **MTB Detected, Rif not detected or R susceptible**_____
B. **MTB Detected, RR**_____

TB enrolment

TB register number: _____MDR register number: _____

Demographic and Socio-economic data

1. Patient's Full Name_____
2. Where do you live (**Residence**)?
   A. Urban
   B. Rural

   2.1. Location: Block number :_____
   Kushet/ketena_____Tabia/Kebelle:_____
   Wereda/district:_____Zone:_____
   mobile Number_____
3. Sex
   A. Male
   B. Female
4. **Age**:_____
5. Marital status?(**for patients≥18)**
   A. Single
   B. Married
   C. Widowed
   D. Divorced
   E. Not applicable

   5.1.If married women **≥18**, number of births/child:_____
   5.2.If married women **≥18**, current condition
      A. Pregnant
      B. Lactating

C. None pregnant and none lactating

6. What is your family size? _____
7. What is your monthly family income (in birr)?
   A. <500
   B. 500-2000
   C. >2000
   D. No means of income
8. What is the highest level of education you have attained at present?
   A. Illiterate
   B. 1-8 grades
   C. 9-12 grades
   D. Diploma and above
9. What is your current occupation?
   A. House wife
   B. Farmer
   C. Self employed
   D. Government employee
   E. Student
   F. No work

Clinical Presentation

10. Would you please tell me if you have one of the following symptoms currently?
    A. Weight loss
    B. Chest pain when breathing or coughing
    C. Coughing up sputum > 2 weeks
    D. Hemoptysis
    E. Shortness of breath
    F. Intermittent fever
    G. Night sweats
    H. Loss of appetite
    I. Fatigue and malaise
    J. Other_____
11. When do you start coughing?_____

TB history and related disease

12. Type of TB case
    A. New (never treated before for ≥1 month)
    B. Relapse
    C. Return after default

D. Failure after treatment

13. Do you have any history of TB patient in your family?

A. Yes
B. No

14. Do you have any close contact with TB patient or chronically coughing patient?

A. Yes
B. No

15. If yes, for how long do you had a contact?_____
16. Have you any close contact with MDR patient?

A. Yes
B. No

17. If yes, for how long do you had a contact?_____
18. History of diabetes mellitus?

A. Yes
B. No

19. HIV status

A. Positive
B. Negative

20. Nutritional status/Anthropometric measurement

A. Weight in Kg_____
B. Height in cm_____

21. Are you currently taking treatment for chronic disease?

A. Yes
B. No

22. If yes, type of treatment/type of chronic disease:_____

Behavior of the patient (≥18 years)

23. Do you have the habit of **alcohol taking**?

A. Yes
B. No

24. If yes, how many time per week?_____

A. 1-2/week
B. 3-5/ week
C. 6-7 /week
D. ≥2 /day

25. What habit do you have?

A. Khat chewing

B. Cigarette smoking

C. Others _____

D. No habit

26. History of prison?
    A. Yes
    B. No

27. If yes, for how long you have been in prison?_____

- **Date of release to regional laboratory_____E.C**

Laboratory data recording format for patients

Laboratory results:

✓ Date of specimen received by THRL: ____/___/___ E.C

Name of investigator: _____Mobile:_____

Laboratory sputum ID: _____, Culture Number: _____

1^0 smear	Media Smear	Blood agar	Capila

TB Culture results

Neg	Pos	conta	NTM	Method

Date reported: ____/____/____Name and Signature of expert _____

Results of *M. tuberculosis* drug susceptibility test

✓**Molecular method used (LPA)**

Result	**1st line drugs**					**2nd line drugs**							Other	
	H	R	S	E	PZA	OFX	LFT	MFX	AMK	CAP	KAN	VIO		

Legend: H= Isoniazid, R= Rifampicin, PZA= Pyrazinamide, E=Ethambutol, S= Streptomycin, Ofx= Ofloxacin, Lft=levofloxacin, Mfx= Moxifloxacin, AMK= Amikacin, CAP= Capromycin, KAN=kanamycin, Vio=viomycin, S = Sensitive, R = Resistant, C = Contaminated, ND = Not done

Comment: _____

Date reported: ____/____/____Name and Signature of expert _____

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