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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,

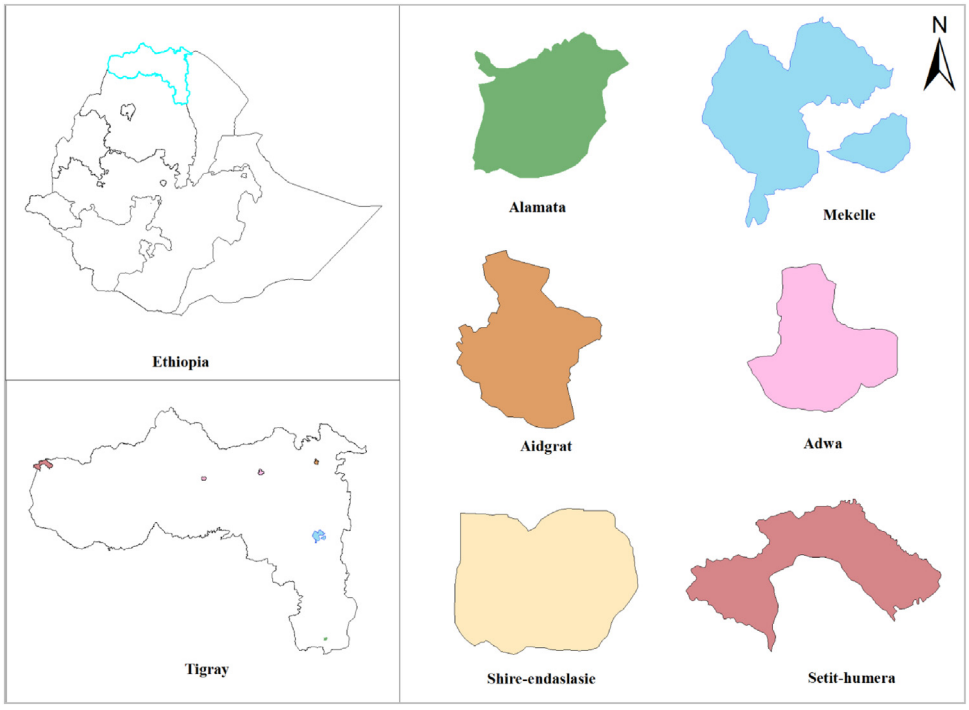


**Fig. 1.** Map of the study area.

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].

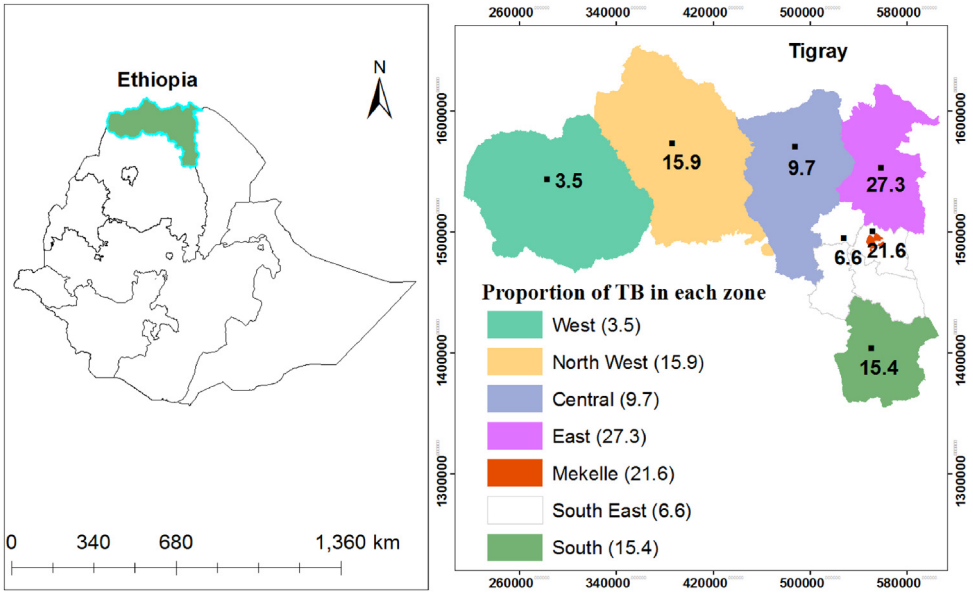


**Fig. 2.** Zonal distribution of the 227 tuberculosis (TB) cases in the Tigray Region, Northern Ethiopia, July 2018 to August 2019. Note: numbers are in percent.

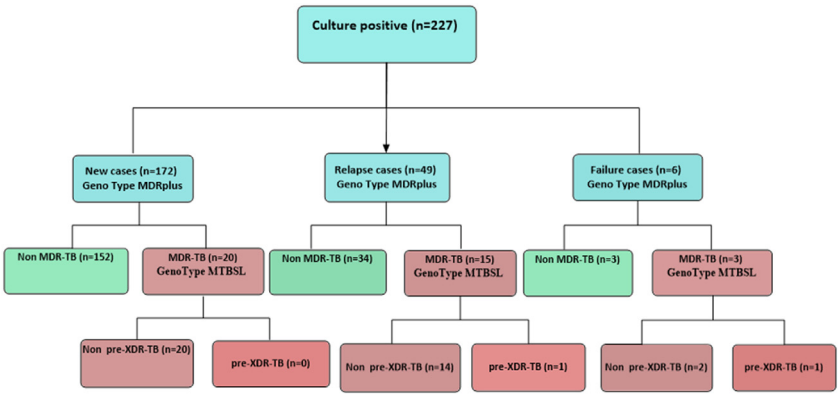


**Fig. 3.** Flow chart of the procedure used to recruit all participants in the study, Tigray Region, Ethiopia, July 2018 to August 2019. MDR-TB, multidrug-resistant tuberculosis; pre-XDR-TB, pre-extensively drug-resistant tuberculosis.

**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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