Utility of line probe assay in detecting drug resistance and the associated mutations in patients with extrapulmonary tuberculosis in Addis Ababa, Ethiopia

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Abstract

Introduction: Molecular tests allow rapid detection of Mycobacterium tuberculosis and drug resistance in a few days. Identifying the mutations in genes associated with drug resistance may contribute to the development of appropriate interventions to improve tuberculosis control. So far, there is little information in Ethiopia about the diagnostic performance of line probe assay (LPA) and the Mycobacterium tuberculosis common gene mutations associated with drug resistance in extrapulmonary tuberculosis. Thus, this study aimed to assess the frequency of drug resistance-associated mutations in patients with extrapulmonary tuberculosis (EPTB) and to compare the agreement and determine the utility of the genotypic in the detection of drug resistance in Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted on stored Mycobacterium tuberculosis isolates. The genotypic and phenotypic drug susceptibility tests were performed using LPA and BACTEC-MGIT-960, respectively. The common mutations were noted, and the agreement and the utility of the LPA were determined using the BACTEC-MGIT-960 as a gold standard.

Results: Of the 151 isolates, the sensitivity and specificity of MTBDRplus in detecting isoniazid resistance were 90.9% and 100%, respectively. While for rifampicin, it was 100% and 99.3% for sensitivity and specificity, respectively. The katG S315Tl was the most common mutation observed in 85.7% of the isoniazid-resistant isolates. In the case of rifampicin, the most common mutation (61.9%) was observed at position rpoB S531L. Mutations in the gyrA promoter region were strongly associated with Levofoxacin and Moxifloxacin resistance.

Conclusion: Line probe assay has high test performance in detecting resistance to anti-TB drugs in EPTB isolates. The MTBDRplus test was slightly less sensitive for the detection of isoniazid resistance as compared to the detection of rifampicin. The most prevalent mutations associated with isoniazid and rifampicin resistance were observed at katG S315Tl and rpoB S531L respectively. Besides, all the fluoroquinolone-resistant cases were associated with gyrA gene. Finally, a validation study with DNA sequencing is recommended.

Keywords
Extrapulmonary tuberculosis, mutation, performance characteristics

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Eastern Mediterranean region (24%), which is more than the global EPTB prevalence (16%). In Ethiopia, EPTB represented 30% of all notified TB cases which is greater than the global average.

Drug-resistant TB is a public health problem and a threat to global TB control programs. Drug-resistant TB is developed when someone is infected with *Mycobacterium tuberculosis* strains that are resistant to at least one anti-TB drug. It can be multi-drug resistant TB (MDR-TB) or extensively drug-resistant TB (XDR-TB). MDR-TB is caused by *Mycobacterium tuberculosis* strains resistant to rifampicin and isoniazid, while XDR-TB is identified as MDR-TB that is resistant to at least one Group A medication and any fluoroquinolone. The Group A drugs are currently levofloxacin or moxifloxacin, bedaquiline, and linezolid. According to the 2020 global TB report, an estimated 3.3% of new TB cases and 18% of previously treated cases develop MDR/RR-TB globally. The prevalence of MDR-TB among new and retreatment cases in Ethiopia is estimated to be 0.7% and 16%, respectively. In Ethiopia, pre-XDR TB was reported by previous studies with 5% to 5.7%. According to recent studies, the prevalence of MDR-TB is 2% and 1.3% in Ethiopia, and that of XDR-TB is 1.3% and 0.7%, respectively.

Conventional drug-susceptibility test (DST) is laborious, time-consuming, and requires the growth of mycobacteria. Therefore, the World Health Organization (WHO) has recommended the use of molecular line probe assay for detecting drug-resistant *M. tuberculosis* directly from smear-positive specimens and cultures-positive isolates. This diagnostic test is based on polymerase chain reaction (PCR) followed by a DNA strip reverse hybridization assay. Drug-resistant mutations in numerous genes have been related to *M. tuberculosis* resistance. Mutations in the *rpoB* gene can explain *M. tuberculosis* resistance to rifampicin (RIF), whereas mutations in the *katG*, and *inhA*, genes are linked to Isoniazid (INH) resistance. Resistance to RIF is mainly associated with mutations in the codon 507 to codon 533 region of the *rpoB* gene (97%). INH resistance is acquired through mutations in the *katG* and *inhA*. The most frequent *katG* mutations (50%-90%) are found at codon 315. Fluoroquinolone resistance is caused by mutations in the *gyrA* and *gyrB* genes. The *gyrA* mutations account for 60% to 70% of all mutations, but *gyrB* mutations are not common. Resistance to second-line injectable drugs (SLID), including amikacin (AMK), kanamycin (KAN), and capreomycin (CAP), is mainly associated with *rrs* gene mutations. Approximately 70% to 80% of CAP resistance and 60% of KAN resistance are caused by the *rrs A1401G* mutation.

For biological and epidemiological reasons, identifying drug resistance-conferring mutations in *M. tuberculosis* in a particular geographical setting is essential. Several studies in Ethiopia investigated drug resistance profiles in *M. tuberculosis* isolates from PTB patients. However, only a few studies have assessed the mutation patterns of *M. tuberculosis* isolates from EPTB samples. A pooled estimate conducted in Ethiopia, revealed that 89.2% of the *katG* mutation was at position S315T1% and 77.5% of the *inhA* mutation was at position C15T. Moreover, 74.2% of *rpoB* gene mutations was observed at position S531L. In another study, all FQs resistance mutations were associated with *gyrA* gene at position D94Y/N. However, the information is very limited. Besides, less is known about the diagnostic performance of line probe assay (LPA) in detecting drug resistance in EPTB isolates in Ethiopia. Hence, this study aimed to assess the frequency of drug resistance-conferring mutations in patients with EPTB and to compare the agreement and determine the utility of the genotypic (LPA) in the detection of drug resistance using the phenotypic (BACTEC-MGIT 960) test as a gold standard.

**Method**

**Study setting, design, and period**

This study was conducted among Mycobacterial isolates collected from 151 patients with EPTB in Addis Ababa, Ethiopia (Figure 1). The isolates were based on the samples collected from EPTB patients in the selected eight public hospitals found in Addis Ababa such as St Paul Hospital, ALERT Hospital, Armed Force Hospital, Black Lion Hospital, Ras Desta Hospital, Zewiditu Hospital, St Peter Hospital, and Yekatit 12 Hospital. A laboratory-based, cross-sectional study was conducted between October 2019 and April 2020 (Figure 2).

**Inclusion and exclusion criteria**

All culture-positive isolates presented with a correct patient identification number and having demographic data were included in this study. NTM and contaminated EPTB isolates were excluded from this study.

**Laboratory analysis**

**Subculturating of stored isolates collection.** The sample collection was conducted from January to August 2017. Isolates were stored in the national TB reference laboratory, Ethiopian Public Health Institute in a deep freeze at a temperature of −80°C with the 7H9 broth base and kept until needed for this study. All stored isolates were sub-cultured in liquid culture media for detection of *M. tuberculosis*.

**Phenotypic drug susceptibility testing.** Phenotypic DST was performed using Mycobacterium growth indicator tubes (MGIT) (BACTECTM MGITTM 960 System). Each MGIT tube was inoculated with 0.8 mL of SIRE supplement. Then, 0.1 mL of the drug solution and 0.5 mL of strain suspension were added. For the drug-free growth control tube, the organism suspension was diluted at 1:100 with sterile saline, and then 0.5 mL was inoculated into the tube and incubated for a maximum of 13 days for all drugs except the PZA drug. The maximum incubation period of the PZA drug is 21 days.
The final concentrations of each drug in liquid medium were: INH 0.1 µg/mL, RIF 1.0 µg/mL, ethambutol 5 µg/mL, STM 1.0 µg/mL, PZA 100 µg/mL, ofloxacin 2.0 µg/mL, CAP 1.25 µg/mL, AMK 1.0 µg/mL, KAN 2.5 µg/mL, moxifloxacin (MOX) 2.5 µg/mL and ethionamide 2.5 µg/mL.

Results were interpreted as follows, a known concentration of drug contained the MGIT along with the specimen, and growth was compared with a drug-free control of the same specimen. At the time when the growth unit (GU) of the drug-free control tube was >400, if the GU of the drug-containing tube to be compared was ≥100, the strain was resistant. If the GU of the drug-containing tube was <100, the strain was susceptible. As a control, the reference strain of *M. tuberculosis* H37Rv was used as control.

**Geno-Type MTBDRplus and MTBDRsl assay.** Line probe assay technology is done in multiple steps. DNA extraction, master mix preparation, PCR, and reverse hybridization were all done in separate rooms during the LPA process. All of the assays (Geno-Type MTBDRplus and MTBDRsl assays) were performed as directed by the manufacturer.

**DNA extraction.** The GenoLyse kit was used to extract DNA according to the manufacturer’s instructions (Hain LifeScience, Nehren, Germany). To kill the bacteria, a 1 ml liquid culture was transferred directly to a conical vial tube. The isolate was then centrifuged at 10,000 g for 5 min. After discarding the supernatant it was resuspended in the pellet in 100 µL lysis Buffer and vortexed. Then it was incubated for
5 min at 95°C in a heat block. After that, 100 μl Neutralization Buffer was added to the lysate and vortexed for 30 s before centrifuging it at 13,000 g for 5 min. For further examination and long storage, the DNA-containing supernatant was transferred to a separate tube. The 5 μL extracted DNA sample was then added to the master mix in another room. The PCR on DNA from culture isolates was performed using the following parameters: 95°C for 15 min, 95°C for 30 s, 58°C for 2 min (10 cycles), 95°C for 25 s, 53°C for 40 s, 70°C for 40 s (20 cycles).

Reverse hybridization. According to the manufacturer’s instructions, hybridization and detection of the amplified product were carried out in an automated TwinCubator. Denaturation of the amplification products was accomplished by mixing 20 μL of amplified products with 20 μL of denaturing reagent (supplied in the kit) for 5 min. After that, 1 mL of pre-warmed hybridization buffer was added, and the operation was carried out at 45°C for 30 min, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin conjugated with alkaline phosphatase and substrate buffer was added. Washing was done well, and all strips were air-dried. To ensure that the test was free of cross-contamination, DNA from H37RV (positive control) and negative control was examined. Only when bands were obtained on MTB complex controls, conjugate controls, and amplification controls in conjunction with the target genes locus controls were the results considered valid.

Results analysis and interpretation. GenoScan® (Hain Lifescience, Nehren, Germany) was used to scan the dried strips and create an automated read-out of the band patterns. The strips were attached to the assessment papers that came with the kit. The read-final out results were manually checked with the naked eye.

Data quality assurance. Internal quality control was analyzed along with the study’s clinical isolates. A known susceptible M. tuberculosis (H37Rv) control and resistant isolates were tested by including them in each test run of DST. Data entry was performed 2 times independently to check inconsistencies. Data cleaning was performed before the final analysis.

Statistical analysis. Descriptive statistics such as frequencies and proportions were used to explain drug resistance patterns and associated mutations. The agreement, sensitivity, specificity, positive, and negative predictive values of LPA compared to the BACTEC MGIT 960 system were calculated for drug resistance. The strength of agreement between the
demographic profile and clinical data of study participants

Out of 151 culture-positive confirmed cases of EPTB isolates, more than half (54.3%) were isolated from males. The mean age of the study participants was 32.3 years ranging from 4 to 78 years. The majority of participants were in the age groups 15 to 64 years. More than half (51.0%) of the study participants attended primary school. About 45% were married and 30.5% were daily laborers. Most (90.7%) of the patients had no previous TB treatment history. About 22.5% and 9.3% of the participants were HIV sero-reactive and had diabetes, respectively (Table 1).

Characteristics of the Mycobacterial isolates

In this study, 151 stored mycobacterial isolates from patients with EPTB were characterized using LPA to identify the mutations associated with drug resistance. Phenotypic DST was used as a gold standard to determine the diagnostic performance of MTBDRplus and MTBDRsl LPA (Figure 2). The majority (99, 65.6%) of the isolates were from lymph node aspirates, followed by pleural fluid (32, 12.2%), peritoneal fluid (8, 5.3%), ascitic fluid (5, 3.3%), cerebrospinal fluid (CSF) (3, 2.0%), pus (2, 1.3%), pericardium fluid (1, 0.7%), and abscess (1, 0.7%) (Figure 3).

Performance of MTBDRplus in detecting RIF and INH resistance

This study analyzed the DST pattern of Mycobacterial isolates by the Geno-Type MTBDRplus assay and the phenotypic DST (MGIT) methods. To test the diagnostic performance of the MTBDRplus in diagnosing RIF and INH resistance, it was compared to the MGIT (gold standard) method. The sensitivity, specificity, positive predictive value, and negative predictive value of the MTBDRplus assay for INH were 90.9%, 100%, 100%, and 98.5%, respectively, while the respective values for RIF were 100%, 99.3%, 93.3%, and 100%, respectively. The kappa agreement for INH between genotypic DST and phenotypic DST was 0.95, while it was 0.96 for RIF. Fifteen and 14
M. tuberculosis isolates were resistant to RIF by the MTBDRplus assay and the phenotypic DST, respectively. Twenty and 22 isolates were resistant to INH in the MTBDRplus assay and the phenotypic DST, respectively. Among the 151 Mycobacterial isolates, 3 (2.0%) M. tuberculosis isolates had discordant results. Of these, two isolates were susceptible to INH in the MTBDRplus assay and became resistant in the phenotypic DST. One isolate was resistant to RIF in the genotyping DST analysis and became susceptible to the phenotype DST. The concordances of the MTBDRplus assay and the MGIT DST for the detection of INH and RIF resistance were 90.9% (20/22) and 100% (14/14), respectively (Table 2).

For all 14 MDR cases, we performed second-line DST using both the Geno-Type MTBDRs/ assay and the phenotypic (MGIT) DST method. Accordingly, three isolates were resistant to second-line drugs in the Geno-Type MTBDRs/ assay where two isolates were resistant to LEV and MOX, while one isolate was resistant to AMK, CAP, and KAN. All these results agreed with the phenotypic DST result.

Table 2. The diagnostic performance of MTBDRplus against phenotypic DST in extrapulmonary specimens (n = 151).

| Geno-Type MTBDRplus assay | Phenotypic MGIT DST result | NPV | Kappa agreement |
|---------------------------|-----------------------------|-----|-----------------|
|                           | Susceptible | Resistance | Sensitivity | Specificity | PPV |                        |
| INH                       | 129 (85.4%) | 2 (1.3%) | 90.9% | 100% | 100% | 98.5% | 0.95 |
| Resistance                | – | 20 (13.2%) |                        |                |     |                   |
| RIF                       | 136 (90.1%) | – | 100% | 99.3% | 93.3% | 100% | 0.96 |
| Resistance                | 1 (0.4%) | 14 (9.3%) |                        |                |     |                   |

RIF: rifampicin; INH: isoniazid; DST: drug susceptibility test; PPV: positive predictive value; NPV: negative predictive value; MGIT: Mycobacterium Growth Indicator Tube; MTBDR: Mycobacterium Tuberculosis Drug Resistance.

Mutations associated with drug-resistance

Based on the MTBDRplus assay, among the 151 isolates, 21 were found to be either RIF or INH resistant. Of the 21 resistant isolates, the rpoB mutation indicated that 15 (71.4%) isolates were resistant to RIF. The RIF-resistant isolates showed mutations at different amino acid positions. In 13 (61.9%) Mycobacterial isolates, the rpoB mutation was at position S315L, and one (4.7%) isolate had a mutation at the H526Y position. Two (9.5%) isolates had mutations in codon 526 to 529 that indicated the absence of wild-type band (WT7), and 13 (61.9%) isolates had mutations in codon 530 to 533 which showed the absence of wild-type band (WT8). Mutations in katG and inhA genes lead to INH resistance. Eighteen (85.7%) isolates had a katG mutation at the S315T1 position, and one (4.7%) had a mutation at the C15T position. Of the INH resistant isolates, a missed wild-type probe was observed only in one isolate at inhA WT1 (Table 3).

For all 14 MDR-TB cases, we performed genotypic DST using the MTBDRs/ assay. Of the 14 mycobacterial isolates,
became resistant to any of the second-line anti-TB drugs. Two isolates showed mutations in the gyrA region with the WT3 mutation and one with MUT3D addition. These mutations correspond to LEV and MOX resistance. Besides, one Mycobacterial isolate had a mutation in the rrs region with WT1 missing and a MUT1 insertion, which corresponds to resistance to AMK, CAP, and KAN (Table 4).

### Discussion

In this study, we assessed the diagnostic performance of the Geno-Type MTBDRplus against the MGIT 960 system for first-line anti-TB drugs. The Geno-Type MTBDRplus assay showed high sensitivity and specificity to RIF (sensitivity 100% and specificity 99.3%). Relatively lower sensitivity and specificity for RIF were observed in studies done in India. The specificity of rifampicin resistance in this study was 99.3%, which is comparable to two previous studies conducted in Ethiopia and another study done in India.21,27,28

In this study, the sensitivity and specificity of LPA for detecting INH resistance were 90.9% and 100%, respectively, which is greater than investigations in India, which had a sensitivity and specificity of 93% and 97%, respectively.24 In this study, INH sensitivity was lower than that reported by previous studies in Ethiopia, 21,28 Uganda,29 and Pakistan.30 According to this study findings, the MTBDRplus assay was unable to detect INH resistance in the two isolates that were detected by the MGIT system. This might be due to an undiscovered mutation in a genomic area that is not targeted by this assay (such as ahpc, kasa, or furA).31 However, the whole genome sequencing is appropriate in this scenario. The MTBDRplus assay had high specificity in detecting INH resistance and RIF resistant isolates in this study, which is consistent with the previous findings.21,28,29

#### Table 3. Frequency of gene mutations associated with rifampicin and isoniazid resistance (n = 151).

| Gene | Band | Mutant probe | Number of strains (n) | Percentage |
|------|------|--------------|-----------------------|------------|
| rpoB | WT1  | 506–509 | – | – |
|      | WT2  | 510–513 | – | – |
|      | WT3  | 513–517 | – | – |
|      | WT4  | 516–519 | – | – |
|      | WT5  | 518–522 | – | – |
|      | WT6  | 521–525 | – | – |
|      | WT7  | 526–529 | 2 | 1.3 |
|      | WT8  | 530–533 | 13 | 8.6 |
| MUT1 |    |    | – | – |
| MUT2A |    |    | 1 | 0.7 |
| MUT2B |    |    | – | – |
| MUT3 |    |    | 13 | 8.6 |
| katG | WT | 315 | 18 | 11.9 |
| MUT1 | S315T1 | 18 | 11.9 |
| MUT2A | S315T2 | – | – |
| inhA | WT1 | –15 | 1 | 0.7 |
|      | WT2 | –8  | – | – |
| MUT1 | C15T  | 1 | 0.7 |
| MUT2 | T8AG  | – | – |
| MUT3A | T8C  | – | – |
| MUT3B | T8A  | – | – |

MUT: mutant; WT: wild type.

#### Table 4. Gene mutations associated with resistance to second-line anti-TB drugs in extrapulmonary specimens (n = 3).

| Strain number | WT | Mutant region or mutation | Mutation type | Gene region or mutation | Drug resistance |
|---------------|----|---------------------------|---------------|-------------------------|----------------|
| 570 | gyrAWT3  | 92–96 | gyrMUT3C | D94G | Levofloxacin and Moxifloxacin |
| 075 | gyrAWT3  | 92–96 | gyrMUT3D | D94H | Levofloxacin and Moxifloxacin |
| 126 | rrsWT1 | 1400 | rrsMUT1 | A1401G | Amikacin, Capreomycin, and kanamycin |

MUT: mutant; WT: wild type.
In this study, we compared the performance of the line probe assay with that of the MGIT 960 system to assess the DST pattern of *M. tuberculosis* in EPTB. The BACTEC MGIT 960 method was considered the “gold standard.” Two (1.3%) isolates gave discordant results for INH between molecular and phenotypic methods. Of these isolates, phenotypic DST revealed resistance to INH, while the genotypic method provided susceptible results. Discordant results between phenotypic and genotypic DST may be due to the fact that not all mutations conferring resistance to anti-TB drugs are included in the LPA assay.

One (0.7%) isolate was shown to be resistant to RIF in the genotype DST but susceptible in the phenotype DST. The discordant results of DSTs for RIF between the genotypic MTBDRplus test and the phenotypic test results were similar to findings reported from Bangladesh and Congo Kinshasa. The MTBDRplus assay detected RIF resistance in one isolate while not detected in MGIT. Being sensitive by phenotypic technique but resistant by genotypic technique might be linked to false RIF resistance, which is caused by a silent mutation that causes the probe to fail to hybridize on a strip and is misinterpreted as RIF resistant.

This study observed the RIF resistance of *rpoB* gene mutations. The most frequently observed mutations on *S315L* and *H526Y* were 61.9% and 4.7%, respectively. This finding was lower than the previous studies reported from Iran, India, and China. However, our finding was similar to the previous study reported from Ethiopia and Sudan in *S315L* and *H526Y* mutations.

In this study, of all the INH-resistant strains, 85.7% had the *S315T1* mutation in the *katG* region. A relatively similar finding was reported by a study conducted in Ethiopia and India, which found 88.0% and 82.94%, respectively. However, this study finding was smaller than the results found in Northern India, where 94.5% of INH resistance isolates had a mutation in the *katG* gene. In addition, previous studies conducted in Ethiopia and India found that mutations in *katG* codons *S315T1* had a greater recorded correlation with INH resistance. Moreover, the study reported from Malawi indicated that the *S315T1* mutation and the mutation in the *inhA* gene that occurred in *C15T* are associated with INH resistance. However, a study in India showed a higher proportion of *C15T* mutations. Similarly, a study reported from Sudan found a *C15T* mutation that resulted in INH resistance.

In this study, a mutation in the *gyrA* codon was found in 14.3% of FQs resistant isolates. This mutation confers resistance to LEV and is associated with low-level resistance to MOX. In this study, *gyrA* mutations were predominantly found to occur in codons 92–96. These most common mutations largely corroborate the findings of the previous study. The predominant *rrs* gene mutation was *A1401G* (7.1%). This is a common mutation reported in previous studies to be associated with high-level resistance to Amikacin, Capreomycin, and kanamycin.

**Conclusion**

The diagnostic performance of the Geno-Type MTBDRplus assay has been confirmed to be highly sensitive and specific for the early detection of MDR-TB. The sensitivity of the MTBDRplus assay for the detection of RIF resistance was high, but the sensitivity was relatively lower for INH resistance detection. However, the kappa agreement between genotypic DST and phenotypic DST was acceptable. Two percent of *M. tuberculosis* isolates had discordant results between the genotypic MTBDRplus test and the phenotypic DST. In INH-resistant strains, 85.7% of strains had *katG S315T1* mutation in the *katG* region, while the most common mutation associated with rifampicin resistance was found at position *rpoB S313L*. Besides, all the fluoroquinolone-resistant cases were associated with *gyrA* gene. The authors recommend a validation study with DNA sequencing.

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The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval
The study obtained institutional ethical clearance from the ethical review committee of the Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University (Protocol Number: DRERC/449/19/MLS). Since the study was conducted using stored clinical isolates, obtaining written informed consent from the study participants was not applicable and it was waived by the ethical committee. The study used a unique study identification number where any of the patients’ identifiers were not used in the entire process. Confidentiality of the results was assured by keeping the documents in a locked area.

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