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ORIGINAL RESEARCH

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

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

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

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

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

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

Introduction

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

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Significant progress has been made in the diagnosis of DR-TB since the introduction of novel molecular diagnostic technologies, such as Xpert MTB/RIF and line probe assays (LPAs), in recent years. As a result, the proportion of patients diagnosed with MDR/RR-TB and placed on treatment has increased significantly.[3] Prior to the availability of these tools, TB/DR-TB diagnosis took weeks to months due to both culture-based detection and drug susceptibility testing (DST),[4] which had repercussions on patient care and the overall TB control program. These include provision of inappropriate treatment to patients, amplification of resistance and continued spread of drug-resistant strains.[5]

Improved knowledge on molecular mechanisms of resistance to anti-TB drugs in the last 20 years has led to the development of commercial genotypic assays for the rapid detection of drug resistance.[6,7] Among these assays, the World Health Organization (WHO) approved the use of LPAs for the detection of *Mycobacterium tuberculosis* complex (MTBC) and common mutations associated with drug resistance to INH, RIF as well as FLQs and SLIDs.[5,8]

Ethiopia is one of the countries that has benefited from the utilization of these technologies. LPAs from the Hain LifeSciences, Nehren, Germany: MTBDRplus and MTBDRsl assays were incorporated into the DR-TB diagnostic algorithm of the national TB control program of Ethiopia.[2,9] In line with this, the national and regional TB reference laboratories have been mandated to perform these assays.

It is well established that a few key mutations are responsible for most of the resistance in anti-TB drugs.[10–12] Besides, the frequency of these mutations differs geographically and this variability affects the efficacy of rapid diagnostic tools in identifying drug resistance.[11,12] Hence, the pool of information on the frequency and pattern of mutations related to drug resistance against key anti-TB drugs from different geographical locations is vital in supporting an evidence-based and patient-centered care, ultimately contributing to the overall TB control effort of the country. However, information on DR-TB, particularly on variants of mutations conferring drug resistance to key anti-TB drugs, is scarce in Ethiopia. Therefore, the current study aimed to determine the frequency and type of mutations conferring drug resistance to INH, RIF, FLQs and SLIDs in isolates obtained from culture-positive pulmonary TB patients from the central, eastern and southeastern Ethiopia.

Materials and Methods

Study Population and Setting

A cross-sectional study was conducted using 224 culture-positive MTB isolates obtained from pulmonary TB patients whose specimens were referred to the regional TB laboratories in Adama and Harar between August 2018 and January 2019, for culture and DST. Adama TB Regional TB Reference Laboratory (ATRRL) serves patients mostly coming from the central and parts of south and eastern regions of Ethiopia that include Amhara, Oromia and Afar. Whereas, Harar Regional TB Reference Laboratory (HRTRL) serves the eastern regions, including Harar, Diredawa, Somali and east Oromia.

Pertinent clinical and sociodemographic information to the study such as treatment history, age, sex and address of patients were collected.

Culture and *Mycobacterium tuberculosis* Complex Isolates

Sputum specimens were processed following standard procedures and inoculated onto the BACTEC™ MGIT™ 960 broth culture system (BD, Sparks, MD, USA). The growth of MTBC was confirmed by Capilia TB-Neo (Tauns Laboratories, Japan) and AFB smear staining.[13] The isolates confirmed as MTBC were then subcultured onto Lowenstein Jensen media and harvested within 3–4 weeks. Two cryo-vials containing 1mL 7H9 liquid medium were prepared and two loopful of colonies of MTBC culture were transferred to each vial, which was later transported to the University of Pretoria's Medical Microbiology Research Department for further testing.

DNA Extraction

DNA extraction was performed for each isolate using the PrimeXtractTM kit (Longhorn Vaccines and Diagnostics, San Antonio, TX, USA). Briefly, 200µL of the culture together with 200µL of 100% ethanol and 200µL lysis buffer were transferred to a 1.5-mL microcentrifuge tube and vortexed. The mixture was then centrifuged and incubated for 5 minutes at room temperature. The entire content was then transferred to an extraction column and centrifuged at 13,000rpm for 60 seconds. After removing the extraction column, the eluate was discarded. The extraction column was then filled with 500µL of wash buffer, centrifuged, and the eluate

was removed. This step was repeated twice followed by washing of the filter with additional 500µL wash buffer and centrifugation of the extraction column to remove trace wash buffer. Finally, the nucleic acid was eluted by 1min of centrifugation at 13,000 rpm using 50µL of preheated (60–70°C) elution solution. The extracted DNA was stored at −20°C for further use.

Genotypic Drug Susceptibility Testing

MTBDR*plus* v2.0. was performed on 224 MTBC culture-positive isolates. MDR/RR-TB isolates were subjected to MTBDR*sl* v2.0. All the procedures that included master mix preparation, amplification and hybridization were performed following the manufacturer's instruction (Hain Lifescience, Nehren, Germany).[14,15] We were not able to perform the test for eight isolates due to reagent shortage.

Interpretation of Results

The evaluation sheet provided with the kit was used to paste the developed strips and determine the resistance status according to the manufacturer's instruction. Four control zones on each strip ensured that the test proceeded smoothly and the reagents performed well. These include conjugate control (CC), which demonstrates the efficiency of the conjugate binding and substrate reaction; amplification control (AC), which excludes mistakes during extraction and amplification and the carry-over of amplification inhibitors; *M. tuberculosis* complex control (TUB), which hybridizes with amplicons derived from all members of the *Mycobacterium tuberculosis* complex (MTBC); and locus controls (*rpoB, katG, and inhA* (MTBDRplus); *gyrA, gyrB, rrs* and *eis* (MTBDRsl)) which detect a gene region specific for the respective locus.

In each strip, there are wild-type (WT) probes and mutant (MUT) probes corresponding to each gene studied. When at least one of the WT bands was missing and the corresponding MUT band appeared, this indicated the presence of a known mutation in the gene of the tested strain, suggesting drug resistance. Additionally, the absence of a WT band without the corresponding MUT band was interpreted as resistance due to unknown mutations. The presence of all WT bands and the absence of all MUT bands were interpreted as isolates susceptible to the drugs tested. The presence of all WT bands together with MUT bands was defined as heteroresistant.

Quality Control

For each run of MTBDR*plus* and MTBDR*sl* assays, molecular grade water and reference strain H37Rv susceptible to all drugs tested were used as negative and positive controls, respectively.

Statistical Analysis

Data were first entered into Excel spreadsheet; cleared, and analyzed using the SPSS statistical software package, V20 (SPSS Inc., Chicago, IL, USA). Frequencies and percentages were used to describe clinical and sociodemographic characteristics as well as drug resistance conferring mutations. Tables and figures were used to present the results.

Ethical Approval

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

Results

Characteristics of the Study Population

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

Drug Susceptibility Testing

GenoType MTBDR*plus*

In this study, we performed genotypic DST using MTBDR*plus* v2.0. on 224 isolates. Of these, 88 (39.3%), 85 (38.0%) were resistant to RIF and INH, respectively. Of those resistant to RIF, 82 (93.2%) were also resistant to INH and 6 (6.8%) were monoresistant. More than a third of the isolates (n=82, 36.6%) were MDR-TB. The majority (n=66, 80.5%) were from previously treated, males (n=51, 62.2%) and those aged between 15 and 34 years (n=50, 60.9%). Furthermore, 37 (45.1%) of those isolates identified as MDR-TB were from eastern Ethiopia, followed by central Ethiopia (n=26, 31.7%). There were 3 (1.3%) INH monoresistant isolates (Figure 1 and Table 1).

GenoType MTBDR*sl*

The MTBDR*sl* v2.0. was performed on 73 MDR-TB and 6 RR isolates. Of these, 77 (MDR=71, RR=6) had an interpretable result and were included in the current study. Further resistance to FLQs and SLIDs was observed only in MDR-TB isolates. Accordingly, 7 (9.1%) were resistant to FLQs and 3 (3.9%) were resistant to SLIDs. All of the FLQ-resistant isolates were from patients' previously treated patients with first-line anti-TB drugs. However, 2 (66.7%) of the SLID-resistant isolates were from new MDR-TB patients. Furthermore, all of the SLIDs and 4 (57.1%) of the FLQ-resistant isolates were from eastern Ethiopia (Figure 1 and Table 1).

Frequency of Mutations Conferring Drug Resistance to INH, RIF, FLQs and SLIDs

Mutations in the *rpoB*

The majority of isolates (n=52, 59.1%) with RIF resistance had mutation at codon S531L followed by D516V (n=13, 14.8%), H26Y (n=8, 9.1%) and H526D (n=3, 3.4%). The remaining (13.6%) had missing WT band without the corresponding MUT band and were reported as unknown mutations. These included WT7 mutation (n=8, 9.1%) and WT8 mutation (n=4, 4.5%) (Table 2).

Mutations in the *katG* and *inhA*

Of the 85 isolates with INH resistance, the majority (n=82, 96.5%) had mutation at codon 315 of the *katG* gene (n=78, 91.8% S315T1 and n=4, 4.7% S315T2), indicating high-level resistance. Mutations in the promoter region of *inhA* gene (which indicate low-level resistance) was observed in the INH monoresistant isolates (n=3, 100%) collected from drug-naïve patients. All had mutation at codon −15 (C-15t). No isolate had a co-mutation at *katG* and *inhA* genes (Table 2).

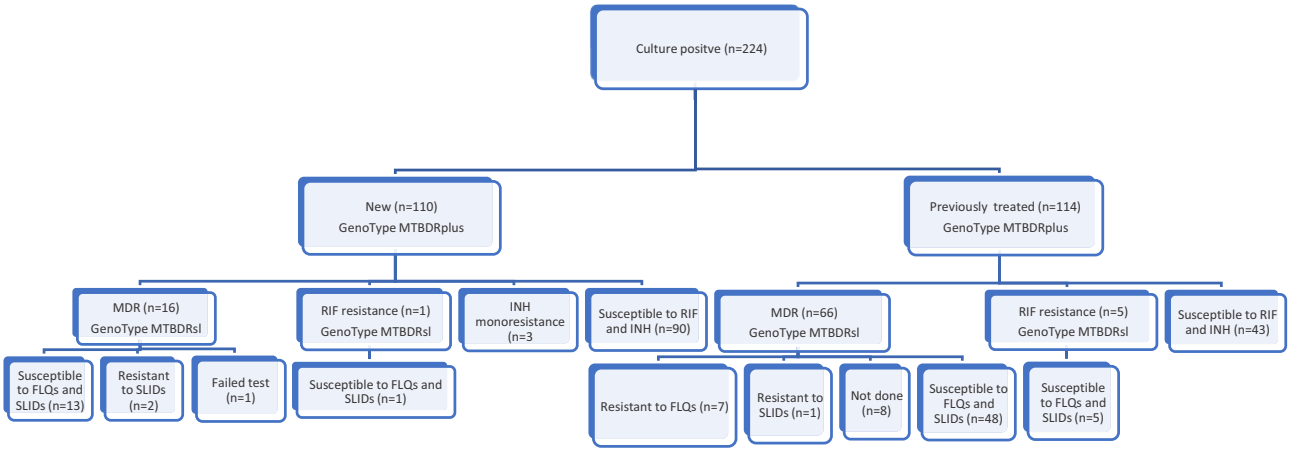

Figure 1 Study population and study flow diagram.

Table 1 Comparison of Patient Characteristics and Drug Resistance Profile

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

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

Table 2 Mutations Conferring Drug Resistance to RIF and INH

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

Abbreviations: RIF, rifampicin; INH, isoniazid; MNR, monoresistant; MDR, multidrug resistant; RR, rifampicin resistant.

Mutations in the *gyrA* and *rrs*

Seven isolates (9.1%) showed mutations that conferred drug resistance to FLQs. The *gyrA* mutation A90V was observed in 3 (42.9%) isolates, whereas mutations D94A, D94G, D94N/Y were each observed in 1 isolate (14.3%). One isolate was heteroresistant where it had all the WT probe together with MUT probe (A90V and D94N/Y) (Table 3). No *gyrB* mutations were observed in the present study. Additionally, three MDR-TB isolates had missing WT bands and without the corresponding MUT band at the *rrs* gene.

Table 3 Mutations Conferring Drug Resistance to FLQs and SLIDs

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

Note: *Heteroresistant.

Discussion

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

Mutations in the 81-bp region (codons 507–533) of the *rpoB* gene harbor over 95% of RIF resistance in MTB isolates and high-level RIF resistance is usually associated with point mutations in 531, 526 and 516 codons.[16] In the current study, among 88 RIF-resistant MTB isolates, the most common gene mutation (59.1%) associated with RIF resistance was at codon S531L. This mutation was reported as a predominant mutation of the *rpoB* gene causing RIF resistance in various studies previously conducted in Ethiopia, which include Jigjiga town (80%),[17] Amhara region (73%),[18] Ethiopia (74.2%),[19] St. Peter's hospital, Addis Ababa, Ethiopia (81.3%),[20] Southwest Ethiopia (82.4%)[21] and Tigray region (70%).[22] In agreement with our finding, higher frequency of mutation at codon S531L was reported in other countries such as Sudan (64.1%), India (62.3%), Iran (66%), Pakistan (64%), and China (58.2%).[23–27]

Although in less frequency, the second most common mutation D516V (14.8%) in this study was previously reported in Ethiopia,[19,20] India[28] and Sudan.[23] However, higher or similar proportion to our findings was reported in Angola (17.2%),[29] India (17.7%),[24] China (10.1%)[27] and Ecuador (28.6%).[30] Eleven isolates (12.5%) showed mutations at codon 526 in which 9.1% were at H526Y and 3.4% at H526D. Comparable findings were reported in Ethiopia[31] and India.[24]

In the current study, 13.6% of the isolates were classified as RIF resistant based on only the lack of WT probe hybridization. This proportion of isolates with unknown mutations is similar to other studies from St. Peter's TB Specialized Hospital, Addis Ababa, Ethiopia (15.8%)[31] and Southwest Ethiopia (14.7%)[32] and a multicenter study in India, South Africa and Moldova (13%).[33]

INH resistance is mainly caused by mutations in the *katG* and *inhA* genes; with 50–95% of the INH-resistant isolates having *kat*G S315 mutations[34] depending on geographical distribution.[35] Similarly, most of the INH-resistant isolates (96.5%) in our study had mutations in the *kat*G gene (S315T1/T2) while the remaining 3.5% had mutations in *inh*A (c15T) promoter region. In agreement with our findings, a meta-analyses study that examined INH conferring mutations[19] reported a prevalence of 95.8% for *kat*G315 mutation and 5.9% for *inh*A promoter region mutation. High-level INH resistance causing mutation (S315T1)[36] was the most frequent (91.8%) in our study and other studies conducted in Ethiopia.[17,18,20,32,37] Furthermore, the *kat*G 315 mutations reported to be frequent in MDR-TB patients[36,38] were exclusively found in MDR-TB isolates in the current study.

Mutations in the *inh*A promoter region which are associated with low-level INH resistance are usually less frequent when compared with *kat*G mutations.[34] In this study, we found only three INH-resistant isolates (all monoresistant) with mutations at codon C15T of the *inh*A promoter gene. In earlier studies from Ethiopia, mutations in *inh*A promoter region were mostly in INH monoresistant isolates.[20,32,38] Other studies also reported no or low proportion of mutation in the *inh*A promotor region.[18,20,31,37,39]

In Ethiopia, the indiscriminate use of FLQs for various indications might have led to the development of drug resistance against these key drugs.[40–42] Mutations in the *gyrA* and *gyrB* gyrase genes especially at codons 90, 91 and 94 of *gyrA* (termed quinoline resistance-determining region, QRDR) are responsible for FQLs resistance in MTB isolates.[43] In this study, *gyrA* mutation at codon A90V was the most common (42.9%) among the FLQ-resistant isolates, which is in agreement with a laboratory-based surveillance study[40] in Ethiopia and a report from Morocco.[44] Supporting our findings, D94N/D94Y was recently reported to be the second most common *gyrA* mutation in Ethiopia.[22,40] Various investigations have indicated that D94G mutation is predominant across the corners of the globe.[43,45–49] In our study, no mutation related to *gyrB* was observed, which is in concordance with previous studies in Ethiopia.[22,40]

Of note, one isolate had a WT probe hybridization and A90V and D94N/Y mutations indicating heteroresistance (coexistence of susceptible and resistant strain in a single specimen).[50] Superinfection or reinfection by a second strain, mixed infection or within host evolution of strains could result in heteroresistance.[51] As an intermediate stage of full

resistance, the detection of heteroresistant mutations is important in guiding the provision of proper treatment regimen.[52,53]

Resistance to SLIDs is mostly associated with *rrs* A1401G mutation.[54] Similarly, the A1401G mutation was reported as the most frequent in Ethiopia.[40] However, in the current study, three isolates had unknown *rrs* mutation that results in cross-resistance to kanamycin, capreomycin and viomycin.[15] It is well known that kanamycin and capreomycin are no more used in the treatment of DR-TB.[55]

Limitations

Our study is not without limitations. The number of isolates included in our study is relatively small and were obtained from patients referred to TB culture/DST laboratories. Furthermore, although proven effective, the sole use of LPAs will not be enough to describe the spectrum of mutations in the country. Hence, the findings of this study might not accurately represent the overall situation in Ethiopia.

Conclusion

The finding of our study showed that canonical drug resistance conferring mutations at *rpoB, katG, gyrA* were the most frequent in RIF-, INH- and FLQ-resistant isolates, respectively. INH monoresistant isolates had mutations exclusively in the *inhA* promoter region. However, a significant proportion of isolates with RIF resistance had unknown mutations that could affect the decision-making in patient management. Hence, the use of better tools such as whole-genome sequencing involving large number of isolates is vital to further elucidate such mutations and predict drug resistance.

Data Sharing Statement

Relevant data pertaining to this study will be provided upon a reasonable request to the corresponding author.

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Disclosure

The authors report no conflicts of interest in this work.

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