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Gene Mutations Conferring Second-Line Drug Resistance in Tuberculosis in Amhara Region: Eight-year Retrospective Study

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Abstract

Background: *Tuberculosis (TB) is an infectious disease that can affect various organs, though it primarily targets the lungs. The global emergence of highly drug-resistant TB strains has significantly undermined treatment and control efforts. Resistance in Mycobacterium tuberculosis is mainly attributed to spontaneous mutations in chromosomal genes. However, data on specific gene mutations associated with second-line drug resistance remain limited in the Amhara Region of Ethiopia.*

Objective: *This study aimed to assess the gene mutations associated with second-line drug-resistant tuberculosis among patients in the Amhara Region, Northwest Ethiopia.*

Method: *A retrospective study was conducted from January 1, 2016, to January 30, 2023. Drug resistance-associated gene mutations were identified using the Genotype MTBDRsl line probe assay. Data were analyzed using SPSS version 26 statistical software. Chi-square test were applied to examine associations between gene mutations and socio-demographic characteristics, with a significance level set at $p < 0.05$.*

Result: *A total of 308 presumptive multidrug-resistant TB (MDR-TB) patients were tested for second-line drug susceptibility. Of these, 165 (53.6%) were male, and the majority (n = 177, 57.5%) were aged 25 - 44 years. HIV co-infection was observed in 41 (13.3 %) patients. Fluoroquinolone (FLQ) resistance due to gyrA mutation at position A90V was identified in 1 (0.3%) isolate. Resistance to second-line injectable drug was observed in 10 (3.2%) isolates, indicated by missing wild-type (WT) bands or mutations in the rrs gene. Among these, 2 (0.6%) had rrs mutations at position A1401G, and 1 (0.3%) at position G1484T. HIV status was significantly associated with FLQ-resistant TB ($\chi^2 = 5.42$, $p = 0.02$), and the year of testing was significantly related to the prevalence of resistance to second-line injectable drugs ($\chi^2 = 13.71$, $p= 0.05$).*

Conclusion: *This study highlights the presence and distribution of gyrA and rrs gene mutations associated with second-line drug resistance in M. tuberculosis isolates from the Amhara Region. The significant association between HIV status and FLQ-resistance underscores the need for integrated TB-HIV management. Routine molecular testing for resistance-conferring mutations is recommended prior to initiating fluoroquinolones and second-line injectable drugs in MDR-TB patients.*

Introduction

The genus Mycobacterium comprises many species, which are typically grouped into three categories: Mycobacterium leprae, the *Mycobacterium tuberculosis* complex (MTBC), and *non-tuberculosis mycobacteria* (NTM)(1). *Mycobacterium tuberculosis (M. tuberculosis)* is a non-motile, non-spore-forming, acid-fast bacterium and a causative agent of tuberculosis (TB)(2).

Tuberculosis is an infectious disease that can affect various parts of the body, although it primarily targets the lungs. Tuberculosis (TB*)* is an infectious disease affecting all parts of the body; primarily affecting the lungs, which in the majority of cases is caused by infection with the intracellular bacterial agent, *M. tuberculosis*(3). Among all bacterial infections, it is the primary cause of human mortality and morbidity worldwide(4).

According to the World Health Organization (WHO), the 2018 report found a high TB burden, particularly in sub-Saharan African countries. Ethiopia was identified as one of the 30 highest TB-burdened countries, with TB remaining one of Ethiopia's leading causes of mortality(5). Once again, Ethiopia is included on the global list of high-burden countries for TB and HIV-associated TB in the period 2021–2025 (6).

Multi-drug resistance tuberculosis (isoniazid and rifampicin resistant TB)(MDR-TB) has caused challenges to global TB control, and it remains a public health crisis and a health security threat (6, 7).

The high prevalence of MDR-TB has hindered the ability of sub-Saharan region, including Ethiopia to control TB effectively(8). Dreadfully, the global rise of extensively drug-resistant tuberculosis (resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one additional Group A drug (bedaquiline or linezolid)) (XDR TB) has deterred efforts in the treatment and control of TB (9). Extensively drug -resistant tuberculosis is caused by *M.tuberculosis,* which is resistant to any fluoroquinolone and at least one additional Group A drug (bedaquiline or linezolid(10). Extensively drug -resistant tuberculosis is tremendously difficult and expensive to treat and has a very high mortality(11).

The mechanism of drug resistance in *M. tuberculosis* primarily arises from spontaneous mutations in its chromosomal genes (12, 13). Resistant MTB to fluoroquinolones (FQs) exhibit mutations within the quinolone resistance-determining region of the gyrA and gyrB genes (14-16)), with most alterations occurring in a short region*(17).* A significant number of mutations identified in the quinolone resistance-determining region of the *gyrA* gene have been associated with resistance to FQs (18). In addition, the rrs and eis genes are involved in second-line injectable drug resistance (associated with resistance to amikacin, kanamycin, and capreomycin) in *MTB*(19, 20). A global study indicated that resistance to second-line injectable drugs was linked to mutations in the *rrs* gene(21), while polymerizations of the *rrs* gene have various adverse effects on treatment success and antibiotic resistance(22).

The WHO claims that detection of drug resistance requires bacteriological confirmation using rapid molecular tests, culture, and sequencing technology before linking the MDR-TB patients to the TIC(6). However, very little data is documented in other parts of Ethiopia about the burden of *gene* mutation on tuberculosis conferring second drug resistance. Therefore, we believe that the Amhara community's lifestyle, culture, and geographic location are different from those of Ethiopian communities, and therefore, the risk of developing drug-resistant TB and gene mutation on MDR-TB may differ. In addition, the burden of *gene* mutation on tuberculosis and the drug resistance profile of *M. tuberculosis* remain largely underexplored in our study area. Therefore, the present study was conducted to detect the frequency of mutant genes conferring second-line drug resistance and its associated factors among MDR TB patients by using the version two -line probe assay MTBDRsl PCR technique.

Method

Study design and setting

A descriptive retrospective study was conducted between January 2016 and January 2023 among MDR-TB patients in the Amhara National Regional State Public Health Institute (APHI), Amhara Region, North West Ethiopia. Amhara National Regional State has 100 hospitals and 917 health centers serving over 25.5 million people. It has 18 MDR-TB treatment initiation centers (TIC), and two facilities offering

the TB culture test (APHI and University of Gondar Comprehensive Specialized Hospital). The APHI is an institute having a BSL-3 TB laboratory, fully furnished to manipulate TB cultures and anti-TB Drug susceptibility tests. It receives samples for TB culture from 11 MDR-TB TICs through an integrated specimen referral system. The integrated specimen referral system covers multiple disease programs, including HIV and TB. Specimen referral services are provided by the Ethiopian Postal Service Enterprise (EPSE) for all health service providers, including peripheral facilities. The postal specimen referral service is a schedule- and phone call-based service that uses vehicles and motorbikes to transport specimens to testing facilities.

Study population

The source population was all MDR-TB patients from the Amhara region whose sputum sample had culture-positive results in APHI

Data collection and laboratory protocols

The socio-demographic characteristics and risk factors were collected from the registration book. Three hundred eighty-eight second-line LPA results were collected from the result reading and reporting template.

Sputum Specimen Collection and Culture Processing for *M. tuberculosis*

All samples were transported to APHI under a cold chain based on the Ethiopian National TB specimen transport systems. Upon receipt, samples were inspected for the minimum sample acceptance criteria(Sample volume greater than 2ml, if the sample was received less than 5 days of collection, if the cold chain was 2-8 ^{o}C, correctly labeled, or leak-proof container) before culture processing (23). Standard procedures were followed during the mycobacterial culturing steps(24). The growth of MTB colonies on LJ culture media was inspected weekly for up to 8 weeks, and the growth was confirmed by Ziehl-Neelsen (ZN) smear staining(25). "Capilia TB-Neo (Tauns Laboratories, Japan)" was utilized to confirm whether the isolates was MTBC or not(26).

GenoType MTBDRsl Assay

M. tuberculosis colonies were scraped from solid media with sufficient growth and added into screw cupped tube containing lysis buffer. The Life Science GenoType MTBDRsl VER 2.0 kit was used to extract the DNA of *M.tuberculosis*(27). The protocol for cultured specimens was selected in multiplex PCR to amplify genes liable for drug resistance such as gyrA, gyrB, and *rrs* genes(27).

The appropriate program was selected for the hybridization in Twin-Cubator. All steps in denaturation, hybridization, conjugation, rinsing, and addition of substrate solution were followed accordingly to see visible bands on the DNA strips. The visible bands on the DNA strip were interpreted according to the instructions given by the HAIN Life Science user manual (27, 28).

Laboratory quality control

The internal quality control (IQC) was tested during each run performed. In each of the LPA tests (MTBDR*sl* VER2.0), sterile water was utilized as the negative control, while the universal reference H37Rv strain, which is sensitive to all anti -TB medicines, was utilized as a positive control. The test reagents and consumables used for testing clinical samples also had records of LOT-to-LOT testing to ascertain performance.

Data analysis and interpretation

Data were checked for completeness, cleaned in Microsoft Excel 2016, and entered into SPSS version 26 software. The results were presented through tables and charts. Means and standard deviations were calculated for continuous variables while the Pearson correlation coefficient was calculated to check the statistical association between the dependent and independent variables using the Chi-square test. P-values less than 0.05 were considered statistically significant.

Result

A total of 308 presumptive MDR-TB patients were tested for second-line drug susceptibility using GenoType MTBDRsl during the study period from 2016 to 2023. Out of this 165 (53.6%) were males. The majority 177(57.5%), of the patients belonged to the 25-44 years age group and 41(13.3 %) were HIV positive. Most 238(77.3%) were new in their TB registration group. About 123 (39.9%) patients were negative for smear microscopy during the LPA test (Table 1).

Table 1: Socio-demographic and clinical information of DR TB Patients in APHI, Ethiopia

Variables		Frequency	Percent
Sex	Female	143	46.4
	Male	165	53.6
Age Category	<25	78	25.3
	25-44	177	57.5
	>44	53	17.2
HIV status	Negative	267	86.7
	Positive	41	13.3
Reason for Test	Diagnosis	83	26.9
	Follow up	225	73.1
Patient TB Registration Group	Failure	29	9.4
	LFU	6	1.9
	New	238	77.3
	Relapse	35	11.4
AFB Microscopy Results	Negative	123	39.9
	Scanty	23	7.5
	P+1	83	26.9
	P+2	42	13.6
	P+3	37	12.0
Year of Examination	2016	9	2.9
	2017	14	4.5
	2018	63	20.5
	2019	69	22.4
	2020	61	19.8
	2021	31	10.1
	2022	43	14.0
	2023	18	5.8

Based on the MTBDRsl assay, out of 308 isolates, 1 (0.3%) had a mutation in the *gyrA* gene of *Mycobacterium tuberculosis*, where the amino acid alanine (A) at position 90 is replaced by valine (V).

Additionally, 10 (3.2%) strains either lacked their WT band or had mutations in the *rrs* genes. Four (1.3%) isolates had missed their wild-type 1 genes, while 3(1%) of isolates had missed their wild-type 2 genes in the *rrs* gene. Two (0.6%) of the isolates had a change from adenine (A) to guanine (G) at nucleotide position 1401, and 1(0.3%) of the isolates had a change from guanine (G) to thymine (T) at nucleotide position 1484 within the *rrs* gene (Table 2).

The chi-square test was used to assess associated factors for the presence of fluoroquinolone-resistant TB and second-line injectable drugs. Patient HIV status was associated with the presence of FLQ-resistant Mtb (X2 =5.42, p-value =0.02) (Table 3). The years of examination were significantly related to the prevalence of second-line injectable drugs (X2=13.71, p-value =0.05) (Table 4).

Table 2: Frequency of gene mutation of *M. tuberculosis* associated with second-line anti-TB drug resistance in 2016 to 2023 (n=308)

Anti-TB determining regions	Type of Gene	Band	Mutant probe	Strains with a missed or mutant gene	
				Number	Percent
Fluoroquinolones resistance-determining regions	*gyrA*	*gyrA* WT1	85–93/92–96	0	0
		gyrA WT2	85–93/92–96	1	0.3
		gyrA WT3	85–93/92–96	0	0
		gyrA MUT1	A90V	1	0.3
		gyrA MUT2	S91P	0	0
		gyrA MUT3A	D94A	0	0
		gyrA MUT3B	D94N/Y	0	0
		gyrA MUT3C	D94G	0	0
		gyrA MUT3D	D94H	0	0
	gyrB	*gyrB* WT	536–541	0	0
		gyrB MUT1	N538D	0	0
		gyrB MUT2	E540V	0	0
Second-line injectable drug resistance-determining regions	*rrs*	*rrs* WT1	1400/1484	4	1.3
		rrs WT2	1400/1484	3	1
		rrs MUT1	A1401G	2	0.6
		rrs MUT2	G1484T	1	0.3
	eis	*eis* WT1	G-37 to -2A	0	0
		eis WT2	G-37 to -2A	0	0
		eis WT3	G-37 to -2A	0	0
		eis MUT1	C-14T	0	0

Table 3: Chi-square test of factors associated with FLQ resistance TB (N=308).

Variables		Frequency No (%)	*FLQ result*		Chi-square (X2)	*p*-value
			S	***R***		
Sex	Female	143(46.4)	138(96.5)	5(3.5)	1.83	0.18
	Male	165(53.6)	163(98.8)	2(1.2)		
Age Category	<25	78(25.3)	77(98.7)	1(1.3)	0.67	0.72
	25-44	177(57.5)	172(97.2)	5(2.8)		
	>44	53(17.2)	52(98.1)	1(1.9)		
HIV status	Negative	267(86.7)	263(98.5)	4(1.5)	**5.42**	**0.02**
	Positive	41(13.3)	38(92.7)	3(7.3)		
Reason for Test	Diagnosis	83(26.9)	82(98.8)	1(1.2)	0.66	0.42
	Follow up	225(73.1)	219(97.3)	6(2.7)		
Patient Registration Group	Failure	29(9.4)	27(93.1)	2(6.9)	3.74	0.29
	LFU	6(1.9)	6(100)	0(0)		
	New	238(77.3)	233(97.9)	5(2.1)		
	Relapse	35(11.4)	35(100)	0(0)		
AFB Microscopy Results	Negative	123(39.9)	122(99.2)	1(0.8)	4.71	0.32
	Scanty	23(7.5)	23(100)	0(0)		
	P+1	83(26.9)	81(97.6)	2(2.4)		
	P+2	42(13.6)	40(95.2)	2(4.8		
	P+3	37(12)	35(94.6)	2(5.4)		
Year of Examination	2016	9(2.9)	8(88.9)	1(11.1)	9.92	0.19
	2017	14(4.5)	13(92.9)	1(7.1)		
	2018	63(20.5)	60(95.2)	3(4.8)		
	2019	69(22.4)	68(98.6)	1(1.4)		
	2020	61(19.8)	61(100)	0(0)		
	2021	31(10.1)	30(96.8)	1(3.2)		
	2022	43(14.0)	43(100)	0(0)		
	2023	18(5.8)	18(100)	0(0)		

Table 4: Chi-square analysis of Factors associated with CAP, KAN, resistance TB (N=308).

Variables		Frequency No (%)	*rrs*		Chi-square (X2)	*p*-value
			S	***R***		
Sex	Female	143(46.4)	138(96.5)	5(3.5)	0.05	0.82
	Male	165(53.6)	160(97)	5(3)		
Age Category	<25	78(25.3)	77(98.7)	1(1.3)	2.42	0.30
	25-44	177(57.5)	169(95.5)	8(4.5)		
	>44	53(17.2)	52(98.1)	1(1.9)		
HIV status	Negative	267(86.7)	258(96.6)	9(3.4)	0.10	0.75
	Positive	41(13.3)	40(97.6)	1(2.4)		
Reason for Test	Diagnosis	83(26.9)	79(95.2)	4(4.8)	0.83	0.36
	Follow up	225(73.1)	219(97.3)	6(2.7)		
Patient Registration Group	Failure	29(9.4)	27(93.1)	2(6.9)	1.42	0.7
	LFU	6(1.9)	6(100)	0(0)		
	New	238(77.3)	231(97.1)	7(2.9)		
	Relapse	35(11.4)	34(97.1)	1(2.9)		
AFB Microscopy Results	Negative	123(39.9)	119(96.7)	4(3.3)	2.45	0.65
	Scanty	23(7.5)	23(100)	0(0)		
	P+1	83(26.9)	81(97.6)	2(2.4)		
	P+2	42(13.6)	40(95.2)	2(4.8)		
	P+3	37(12)	35(94.6)	2(5.4)		
Year of Examination	2016	9(2.9)	9(100)	0(0)	**13.71**	**0.05**
	2017	14(4.5)	14(100)	0(0)		
	2018	63(20.5)	59(93.7)	4(6.3)		
	2019	69(22.4)	67(97.1)	2(2.9)		
	2020	61(19.8)	61(100)	0(0)		
	2021	31(10.1)	31(100)	0(0)		
	2022	43(14.0)	39(90.7)	4(9.3)		
	2023	18(5.8)	18(100)	0(0)		

Discussion

For TB to be effectively treated and controlled, gene changes on MTB that confer drug resistance must be regularly surveyed and continuously monitored. To develop targeted TB management methods in the region, it is essential to comprehend local gene changes in TB that result in treatment failure. Therefore, this eight-year retrospective data was collected to assess fluoroquinolone and second-line injectable drug resistance-determining genes in *M. tuberculosis*.

In this study, among the 308 isolates, the *gyrA* mutation indicated that 1(0.3%) was resistant to fluoroquinolones, which is lower than the frequency of *gyrA* gene mutation in South Africa, 1.3% (29), and Morocco 2.22% (30). Even a very high prevalence of *gyrA* gene mutations was documented in studies in China, with 62% (31), Russia 94.7% (32), Uzbekistan 89% (33), and India, 39% (34). Fluoroquinolones resistance-determining regions, such as codon D94G, A90V, and S91P of *gyr*A in fluoroquinolone-resistant TB were frequently reported in several studies (35-40). In our results, the FLQ resistance gene in *gyrA* was positioned at A90V, which is in agreement with a laboratory-based surveillance study in Ethiopia(41), Central, Southeastern, and Eastern Ethiopia (36), and a report from Morocco(42). These differences may be due to variations in MTB stain, study period, geography, study population, study design, and methods.

Our study found no isolates harbored mutations in the *gyrB* and eis genes. Likewise, no mutations were detected at *gyrB* in a previous study in Northwest Ethiopia (43) and Central, Southeastern, and Eastern Ethiopia(36). Consistent with our findings, there was no *gyrB* mutation in tuberculosis strains in the Moroccan study(44). In contrast, the frequency of *gyrB* gene mutation was 4% (45) in France and 3.3% in South Africa (46), 7.7%) in Germany (33), 7.7% in Russia (47), and 5.9% in China(48). These variations in *gyrA* and *gyrB* gene mutations may be due to the higher MDR TB strain in China, Russia, and India, and higher pre-XDR and XDR TB circulating in their populations. Based on the WHO global report, these countries have the largest numbers of XDR TB cases globally (5, 6), which supports the above statement. Additionally, this discrepancy might be due to the significant geographical variation of TB lineage distribution, as studies showed that the Central African and East African-Indian lineages were confined to East Africa, while the East Asian lineage was predominantly found in Southern Africa (49, 50). Furthermore, it was revealed that Mycobacterium tuberculosis Sub-Lineage 4.2.2/SIT149 was the predominant Drug-Resistant Clade in our study area, which could be the reason for the discrepancies in the outcomes(51).

Scholars showed that mutations in several genetic loci of Mtb strains had been implicated in the development of resistance to second-line injectable anti-TB drugs, such as resistance to KAN, AMK, and CAP (52, 53). Based on our results, 10 (3.2) % of strains either missed their WT band or mutated at the rrs genes, indicating second-line injectable drug resistance, which was similar to the studies in Myanmar (3.3%)(54). Disparity, the prevalence of *rrs* gene mutation was lower in Kenya (1.5%) (55) and no mutation on the rrs gene in the Moroccan study(56).

On the other hand, the frequency of rrs gene mutation in our study is lower than the frequency of *rrs* gene mutation in China (88.5%) (57), (94.9%) in South Africa (58), (92%) in Latvian study(59), (53.33%) in Indian (60), 25% in Atlanta(61), (44.1%) in Thailand(62), and (89.65%) strains in India(63). These differences may be due to the variation in the prevalence of pre-XDR and XDR TB among the countries, variations of tuberculosis strain in the study areas, the study period, and the sample size. Moreover, the method used to assess the *rrs* gene mutation may cause a difference in the prevalence of *rrs* gene mutation. For instance, Kumari, et al. (63) used the primers for multiplex allele-specific PCR assays, designed to identify *gyrA* D94G and *rrs*A1401G mutations, and the agarose gel images of PCR products were applied to detect gene mutation. In the present study; commercially available Genotype MTBDRsl kits were used to interpret any gene mutation. The researcher also stated that the accuracy of the result depends largely on the strength of the association between a specific mutation and the phenotypic resistance of the isolates(64), which supports our explanation for the discrepancy.

In the present study, 2 (0.6%) Mycobacterial isolates had the rrs mutation at position A1401G, and 1 (0.3%) at the G1484T. Similarly, the A1401G mutation was reported as the most frequent codon in Ethiopia(41) and a systematic review also noted that resistance to second-line injectable drugs is mostly associated with *rrs* A1401G mutation(64).

Scholars have found that diabetic mellitus, age, previous treatment history, and HIV are among the risk factors for anti-tuberculosis resistance (65-68). However, in our data, we observed that patient HIV status was the only factor associated with the presence of second-line injectable drug-resistant TB (X2 =5.42, p-value =0.02). Similarly, the positive association between HIV and anti-tuberculosis resistance was observed in a study(69). Inconsistent with our study, HIV serostatus was not associated with fluoroquinolone resistance in the United States population(70). Eldholm, V., et al. conclude in their study that HIV co-infection does not significantly affect the mutation rate of Mtb within patients and is not associated with the emergence of resistance(71). This variation may be due to differences in the assessment method, the study population, the prevalence of HIV in the community, and the study periods.

Conclusion

In conclusion, the present study shows the distribution of *gyrA* and *rrs* gene mutations in this study area. Moreover, patient HIV status was associated with the presence of FLQ-resistant TB. Thus, MDR-TB patients in the study area should be monitored for gene mutation before the initiation of fluoroquinolone and second-line injectable drugs.

Data availability statement: All the data sets analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical consideration: The study was approved by the Ethical Review Committee (ERC) of the Health Research Development Directorate of Amhara regional state with reference number NoH/R/T/T/D/07/88.

Participant information was treated confidentially, and specimens collected were used solely for the study's intended purposes. All procedures in this study were conducted by the amended Declaration of Helsinki(72).

Author contributions: GB, HG, and YG contributed to conceiving the research idea, data collection, and data analysis. GB, MG, AT, TM, TB and BB contributed to the conception of the research idea, method rationalization, data analysis, interpretation of results, evaluation of scientific content, and manuscript preparation. GB, YG, MG, AA, TM, AT, and BB were also involved in reviewing and editing the manuscript. All authors had read and approved the final manuscript for submission.

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Abbreviations: APHI: Amhara Public Health Institute, CLSI: Clinical and Laboratory Standards Institute, FLQ: Fluoroquinolone, LPA: Line Probe Assay, MDR-TB: Multi-Drug Resistance Tuberculosis, XDR-TB: Extensively Drug Resistance Tuberculosis, WT: Wild Type

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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