Infection and Drug Resistance

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ORIGINAL RESEARCH

Mycobacterium tuberculosis Sub-Lineage 4.2.2/ SIT149 as Dominant Drug-Resistant Clade in Northwest Ethiopia 2020–2022: In-silico Whole-Genome Sequence Analysis

Daniel Mekonnen[1,2], Abaineh Munshea[2,3], Endalkachew Nibret[2,3], Bethlehem Adnew[4], Hailu Getachew[5], Amiro Kebede[5], Ananya Gebrewahid[5], Silvia Herrera-Leon[6], Aranzazu Amor Aramendia[7], Agustín Benito[8], Estefanía Abascal[6], Camille Jacqueline[6,9], Abraham Aseffa[4], Laura Herrera-Leon[6,10]

[1]Department of Medical Laboratory Sciences, School of Health Science, College of Medicine and Health Sciences, Bahir Dar University, Bahir Dar, Ethiopia; [2]Health Biotechnology Division, Institute of Biotechnology, Bahir Dar University, Bahir Dar, Ethiopia; [3]Department of Biology, Bahir Dar University, Bahir Dar, Ethiopia; [4]Armauer Hansen Research Institute, Addis Ababa, Ethiopia; [5]Amhara Public Health Institute, Bahir Dar, Ethiopia; [6]National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain; [7]Fundación Mundo Sano, Madrid, Spain; [8]National Center of Tropical Medicine, Institute of Health Carlos III, Centro de Investigación Biomédica En Red de Enfermedades Infecciosas, Madrid, Spain; [9]European Public Health Microbiology Training Programme, European Centre for Disease Prevention and Control, Stockholm, Sweden; [10]CIBER Epidemiologia y Salud Publica, Madrid, Spain

Correspondence: Daniel Mekonnen, Email nigusdaniel@gmail.com

Introduction: Drug resistance (DR) in *Mycobacterium tuberculosis* complex (MTBC) is mainly associated with certain lineages and varies across regions and countries. The Beijing genotype is the leading resistant lineage in Asia and western countries. *M. tuberculosis* (Mtb) (sub) lineages responsible for most drug resistance in Ethiopia are not well described. Hence, this study aimed to identify the leading drug resistance sub-lineages and characterize first-line anti-tuberculosis drug resistance-associated single nucleotide polymorphisms (SNPs).

Methods: A facility-based cross-sectional study was conducted in 2020–2022 among new and presumptive multidrug resistant-TB (MDR-TB) cases in Northwest Ethiopia. Whole-genome sequencing (WGS) was performed on 161 isolates using Illumina NovaSeq 6000 technology. The SNP mutations associated with drug resistance were identified using MtbSeq and TB profiler Bioinformatics softwares.

Results: Of the 146 Mtb isolates that were successfully genotyped, 20 (13.7%) harbored one or more resistance-associated SNPs. L4.2.2.ETH was the leading drug-resistant sub-lineage, accounting for 10/20 (50%) of the resistant Mtb. MDR-TB isolates showed extensive mutations against first-line anti-TB drugs. Ser450Leu/(tcg/tTg) for Rifampicin (RIF), Ser315Thr/(agc/aCc) for Isoniazid (INH), Met306Ile/(atg/atA(C)) for Ethambutol (EMB), and Gly69Asp for Streptomycin (STR) were the leading resistance associated mutations which accounted for 56.5%, 89.5%, 47%, and 29.4%, respectively. The presence of both clustered and non-clustered drug resistance (DR) isolates indicated that the epidemics is driven by both new DR development and acquired resistance.

Conclusion: The high prevalence of drug-resistant TB due to geographically restricted sub-lineages (L4.2.2.ETH) indicates the ongoing local micro epidemics. The Mtb drug resistance surveillance system must be improved. Further evolutionary analysis of L4.2.2.ETH strain is highly desirable to understand evolutionary forces that leads L4.2.2.ETH in to high level DR and transmissible sub-lineage.

Keywords: *Mycobacterium tuberculosis*, L.4.2.2, SIT149, drug resistance, Ethiopia

Introduction

Antimicrobial resistance is a hidden global pandemic that shattered over 4.9 million people in 2019 alone, and the burden is highest, mainly in low-resource settings.[1] Drug-resistant tuberculosis (DR-TB) caused by *Mycobacterium tuberculosis (Mtb)* complex (MTBC), which is resistant to one or more anti-TB drugs, is a leading global public health challenge.[2]

Received: 30 July 2023
Accepted: 9 October 2023
Published: 26 October 2023

Multidrug resistant TB (MDR-TB) refers to TB patients infected with MTBC strains that is resistant to rifampicin (RIF) and isoniazid (INH).[3] The MDR-TB mortality rate was estimated to be over 20%.[4]

Globally, an estimated 450000 incident cases of MDR-TB or rifampicin-resistant TB (MDR/RR-TB) have been reported by 2021, with a 3.1% increase since 2020.[5] India (26%), the Russian Federation (8.5%) and Pakistan (7.9%) shared the highest global DR-TB case load.[5] Based on a review of 24 articles in Ethiopia, the prevalence of ionized (INH) monoresistance and any INH resistance was gauged at 6.2% and 15.6%, respectively. Similarly, the RIF monoresistance and any RIF resistance was estimated at 2.3% and 9.7%, respectively. Further, the overall pooled prevalence of MDR TB was 10.8%.[6]

Unlike other prokaryotes in which drug resistance (DR) is mainly mediated by mobile genetic elements, Mtb DR is mediated by spontaneous mutations in the form of single nucleotide polymorphisms (SNP) in various regions of the bacterial genome.[7,8] In addition, several intrinsic and extrinsic bacterial factors play a role in the evolution of DR. Some of these factors include delayed diagnosis, inadequate treatment, poor drug quality, patient noncompliance and pharmacodynamics, persistence, dormancy, cross-resistance, antagonistic epitasis, and biofilm formation.[9] Surprisingly, DR-TB has mainly been reported in countries with well-functioning health systems and good treatment adherence policies. This suggests that drug resistance in MTBC is determined by the strain genetic background besides patient and programmatic related factors.[8] The MTBC genotypes were geographically structured.[10] Hence, the MTBC responsible for DR-TB varies across regions and countries.[11–18]

The Beijing genotype is the leading global DR-TB lineage[19] with extensive widening in the Eurasia landmass.[20] Different sub-lineages, including L4.2.2/SIT149, T3-ETH, Haarlem, CAS, EAI5, and L3/SIT25, have been identified in Ethiopia, showing different levels of resistance.[21–24] It was noted that L4.2.2. ETH/SIT149 is known by different names in Ethiopian literatures. Some of these included Ethiopia_3,[25] NW-ETH3,[26] L4.2. ETH,[27] SIT-149.[28] Mtb isolates with sympatric host associations are more likely to be MDR than allopatric genotypes.[29]

While Africa is viewed as an evolutionary foci for the most recent common ancestor of MTBC, Asia is recognized as the evolutionary center for DR-Mtb. The World Health Organization (WHO) developed a comprehensive catalogue of MTBC mutations associated with first- and second-line anti-TB drugs using 38215 isolates with both phenotypic and genotypic data collected from 41 countries.[30,31] Briefly, a mutation in *rpoB*, which encodes the β-subunit of RNA polymerase determines RIF resistance.[31] Mutations in catalase-peroxidase (*katG)* and 2-trans-enoyl-acyl carrier protein reductase (*inhA*)-encoding genes are associated with INH resistance.[32] While mutation at *embCAB* (for arabinosyl transferase) leads to Ethambutol (EMB) resistance,[33] mutation at *pncA* which encodes for pyrazinamidase/nicotinamidase is associated with Pyrazinamide (PZA) resistance.[34] Streptomycin (STR) resistance is determined by genes encoding ribosomal protein S12 (*rpsL*), 16S rRNA (*rrs*) and 7-methylguanosine (m7G) methyltransferase (*gidB*).[35]

The major lineages or sub-lineages responsible for most Mtb drug resistance in Ethiopia were not highlighted before. Furthermore, first-line anti-TB DR-related SNPs are poorly characterized. This research is an extension to our previously published article.[36] While the published article[36] focused on genomic diversity and transmission dynamics, the present article ascertained the leading drug resistance sub-lineages, explored and characterized mutations associated with drug resistance to first-line anti-TB drugs.

Materials and Methods

Study Design, Population, Period and Setting

This facility-based cross-sectional study was conducted using two groups of (newly diagnosed and presumptive MDR TB) isolates. The first group of isolates were obtained from new (with no previous history of anti-TB treatment) PTB and tuberculous lymphadenitis (TBLN) cases at Felege-Hiwot Comprehensive Specialized Hospital (FHCSH), Bahir Dar, Han and Shum-Abo Health Centers from February 2021 to June 2022. The second group of Mtb isolates obtained from presumptive MDR-TB patients (previously treated for TB, had failed anti-TB treatment, or had been in contact with MDR-TB patients), collected from June 2020 to June 2022, and stored in the Mycobacterium reference laboratory at the Amhara Public Health Institute (APHI).

Mycobacterium tuberculosis Culture and Colony Extraction

The stored isolates were re-grown using two MTBC growth media: Löwenstein-Jensen (LJ) medium[37] and mycobacterium growth indicator tube (MGIT) BACTEC 960 detection system.[38] When both LJ and MGIT-960 were positive with sufficient colonies, the colony was extracted from LJ; otherwise, MGIT-960 was used. From the day of positivity, the liquid culture was maintained at 37°C for an additional 7 days to enrich the MTBC population and make the MTBC cells aggregate and plainly visible in the transparent media. One milliliter of MGIT-960 medium containing MTBC aggregates was placed in 1.5 milliliter screw-cap vials. The MTBC colonies on LJ media were harvested using a sterile plastic loop and transferred to 1.5-mL screw-cap vials containing 1 mL of distilled water. Colonies were inactivated by heating at 95°C for 30 minutes.[39] Heat-inactivated MTBC was transported to the National Center of Microbiology, Institute of Carlos III (ISCIII), Madrid, Spain for DNA extraction and whole-genome sequencing (WGS).

Mycobacterium tuberculosis DNA Extraction

The DNA extraction was carried out using manual NZY Tissue gDNA isolation kit (nzytech genes and enzymes, Lisboa, Portugal)[40] and a robotic Maxwell RSC cultured Cell DNA extraction kit (Promega Biotech Ibérica S.L.).[41] The following adjustments were made to the manufacturer's instructions for DNA extraction using the NZY Tissue Kit. Heat-inactivated MTBC isolate was centrifuged for two minutes at 11000rpm. Following the removal of the supernatant, 180µL of pre-aliquoted lysis buffer and 60µL of reconstituted lysozyme (20 mg/mL) were added. The mixture was then vortexed and incubated at 37°C for 3h. After addition of 75ul of sodium dodecyl benzene sulfonate (SDS) 10x and 20ul µL proteinase K (20 mg/mL), the mixture was vortexed and incubated overnight at 56°C. The samples were subjected to the same pretreatment prior to DNA extraction using the robotic Maxwell RSC DNA kit-based extraction. After DNA extraction, a fluorometer (Promega Corporation) was used to determine the concentration of double-stranded DNA. NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to determine purity at 260/280 absorbance ratio. A DNA quantity greater than 1ng/µL was used as the cutoff criterion for library preparation.

Library Preparation and Sequencing

The Nextera DNA Flex Library Prep Workflow was used to prepare the input DNA library, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The Illumina NovaSeq 6000 was used for sequencing.[42]

Bioinformatics Analysis

The Bioinformatics Unit at the ISCIII performed quality control analysis using fastp; an ultra-fast all-in-one FASTQ preprocessor (QC, adapters, trimming, filtering, splitting); fastQC v.0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic v.0.36 software.[43] The reads were assembled using SPAdes v.3.8.0[44] and the quality of genome assembly was assessed using QUAST software.[45]

MTBseq 1.0.3 bioinformatics tool[46] was used to map the genome against RefSeq (GCF_003287165.1_ASM328716v1). Briefly, mapping was performed using the Burrows-Wheeler Aligner-Maximum Exact Match (BWA-MEM) algorithm. Sorting, indexing, deleting probable PCR duplicates, and removing temporary files were accomplished using Sequence Alignment/Map tools (SAM tools). The Genome Analysis Toolkit (GATK) was used to recalibrate base calls and real readings around insertions or deletions. Subsequently, strain and group calling, variant calling/discovery, and parsing were conducted. Genome annotation was performed using Prokka.[47]

Variants are indicated using four reads mapped in each forward and reverse orientation, at 75% allele frequency, and at least four calls with a Phred score of at least 20. The final sample-specific module performs phylogenetic classification of the input sample(s) using Homolka et al and Coll et al SNP-based typing.[48,49] In SNP-based barcoding, variant subsets are automatically generated and filtered for the repetitive regions and resistance-associated genes.[46] Molecular drug resistance was determined using MtbSeq 1.0.3[46] Mykrobe predictor[50] and a TB-profiler (Galaxy Version 4.1.1+ galaxy).

Multiple sequence alignment (MSA) was performed against H37Rv (GenBank accession number NC_000962.3) using a fast Fourier transform (MAFFT).[51] SNP-based phylogenetic reconstruction was then performed using randomized accelerated maximum likelihood (RaxML) with the general time reversal (GTR) model and a bootstrap value of

1000.[52] Ancient *M. tuberculosis* (Anc Mtb) was used as the root. The tree was edited using FigTree (https://github.com/rambaut/figtree/releases). The sub-lineages, resistance SNPs and associated drugs were summarized using tables and figures.

Results

Molecular Epidemiology of Drug Resistant *M. tuberculosis*

Of the 146 Mtb genotypes (66 from TBLN and 80 from PTB) successfully identified using the WGS technique,[36] 20 (13.7%) became resistant to at least one anti-TB drug. Of these, 18 were obtained from presumptive MDR-PTB cases and rest two were obtained from new PTB and TBLN cases (one from each). The proportion of MDR-TB was 17/146 (11.6%). Of the 20 resistant isolates, 10 (50%) belonged to L4.2.2. ETH, followed by Delhi-CAS (n = 6; 30%). The remaining four drug-resistant sub-lineages were L4.1.1.1/X-type, L4.3.4.1/LAM, L4.1.2.1/Haarlem, and L4.8/T (Figure 1). Further calculation of the proportion of drug resistance with respect to their sub-lineage confirmed that L4.2.2.ETH was still the leading drug-resistant isolate, 10/29 (34.5%). Similarly, the proportion of drug resistance in L3 was estimated at 14% (6/43). The phylogenetic tree depicts the distribution of DR Mtb sub-lineages (Figure 1).

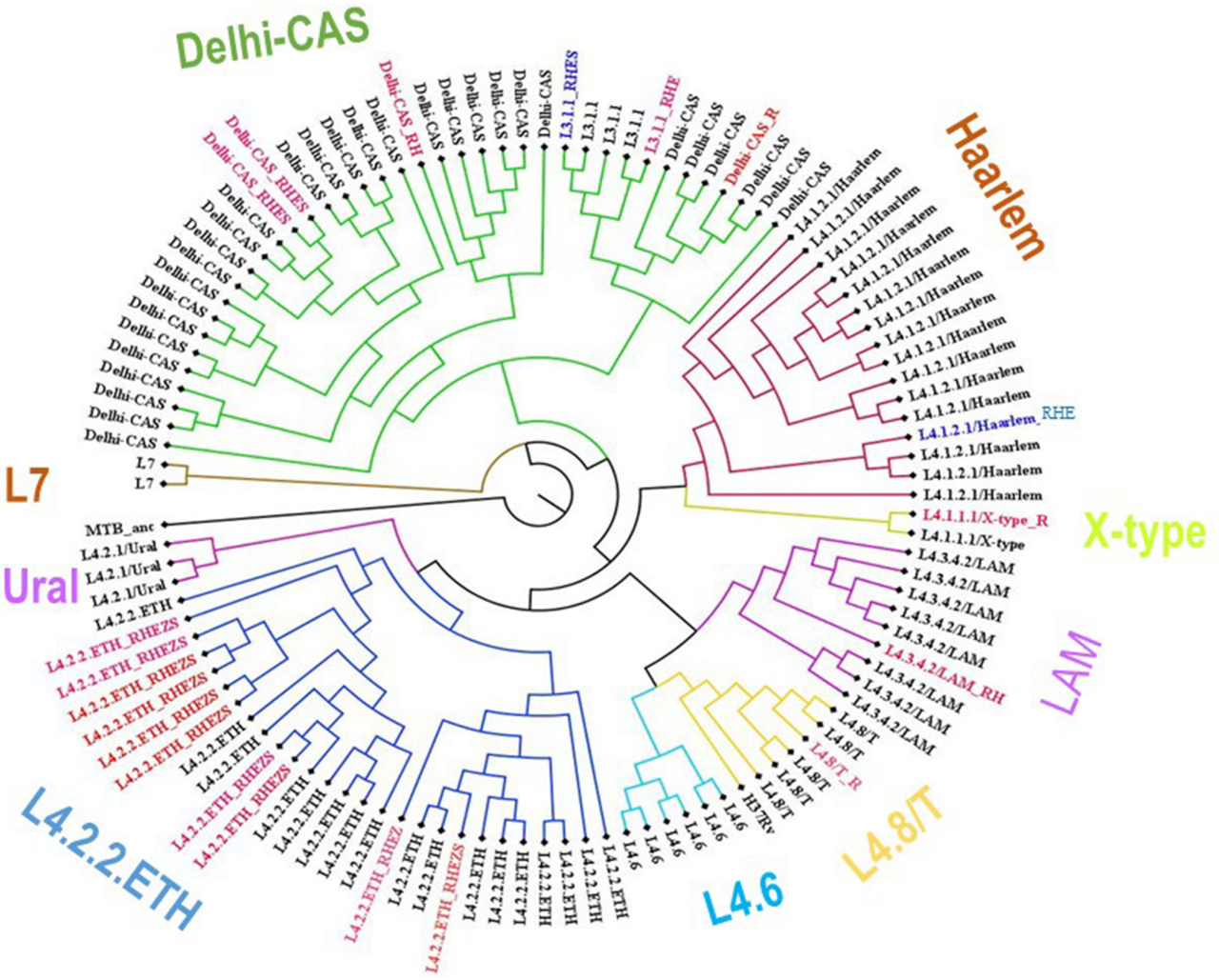

Figure 1 The *M. tuberculosis* phylogeny showing the drug resistant isolates and associated anti-TB drugs, Northwest Ethiopia, 2023.
Notes: Taxa labeled with red (18 sub-lineages) are drug-resistant isolates from previously treated PTB and taxa labeled with blue (2 sub-lineages) are drug-resistant isolates from new TBLN and PTB cases (one from each). The single letter drug names next to sub-lineages at the node of the tree are the resistant anti-TB drugs. R: rifampicin (RIF), H: isoniazid (INH), E: ethambutol (EMB), S: streptomycin (STR), Z: pyrazinamide (PZA).

Drug Resistance Profile of *M. tuberculosis* Sub-Lineages

Of the 20 isolates with one or more drug resistance mutations, 17 were MDR and three were RIF mono-resistant. No INH monoresistance and second-line anti-TB drug-resistance-associated mutations were identified. All ten isolates from L4.2.2. ETH demonstrated high-level MDR (MDR+: resistant to additional first-line drugs). A closer look at the drug resistance profile of each isolate showed that five L4.2.2. ETH/SIT149 sub-lineages were resistant to RIF (R), INH (H), ethambutol (EMB, E), pyrazinamide (PZA, Z) and streptomycin (STR, S); RHEZS. Two L4.2.2.ETH/SIT149 sub-lineages were resistant to RHEZ. Similarly, two L4.2.2.ETH sublineages harbored resistance mutation against RHES, and only one L4.2.2.ETH contained resistance determining mutation to RHE. Among the six lineage 3 resistant isolates, five were MDR (2 resistant for RHES, 2 resistant for RHE and one resistant for RH) and one was RIF monoresistance. All seven PZA-resistant isolates were from L4.2.2. ETH sub-lineages. In addition, the degree of resistance was low among generalist EA sub-lineages (X-type, Haarlem, LAM, and L4.8/T) compared to specialist sub-lineages (L4.2.2. ETH and some members of L3) in the present study (Table 1).

Table 1 First-Line Anti-TB Drugs Resistant Sub-Lineages and Associated Amino Acid Substitutions /Mutation, Northwest Ethiopia, 2023

Sub-Lineages	Rifampicin-RIF/R	Isoniazid-INH/H	Ethambutol-EMB/E	Pyrazinamide-PZA/Z	Streptomycine-STR/S	#sub-lineages/ Resistant to
	rpoB	*katG*	*embB*	*pncA*	*rpsL/gibB*	
Delhi-CAS	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Met306Ile (atg/atA)		-/ c.386delG	1/RHE
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Arg (agc/agA) Ser315Thr (agc/aCc)	Gln497Arg(cag/cGg)	Val139Gly (gtg/gGg)	-/ c.102delG	1/RHEZ
L4.1.1.1/ X-type	Ser450Leu (tcg/tTg)	-	-	-	-/-	1/R
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	p.Ser347Ile		-/ p.Gly69As	1/RHE
L4.2.2.ETH	His445Tyr (cac/Tac) His445Arg (cac/cGc)	Ser315Thr (agc/aCc)	Met306Ile (atg/atC)	c.66_67insT	-/ p.Gly69Asp	2/RHEZS
Delhi-CAS	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	-	-	-/-	1/RH
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Gly406Ala (ggc/gCc)	Val180Phe (gtc/Ttc)	Lys88Thr/ Gly69Asp	1/RHEZS
Delhi-CAS	Leu430Pro (ctg/cCg)	-	-	-	-/-	1/R
L4.3.4.1/LAM	His445Asn (cac/Aac)	Ser315Thr (agc/aCc)	-	-	-/-	1/RH
L4.8/T	Ser431Thr (agc/aCc)	-	-	-	-/-	1/R
L4.1.2.1/Haarlem	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Met306Ile (atg/atA)	-	-/-	1/RHE
L4.2.2.ETH	Gln432Pro Ser441Leu	Ser315Thr (agc/aCc)	Ser297Ala (tcg/Gcg)	-	-/87delC	1/RHES
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Arg (agc/agA) Ser315Thr (agc/aCc)	Gln497Arg(cag/cGg)	Val139Gly (gtg/gGg)	-/102delG	1/RHEZS
Delhi-CAS	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Met306Val(atg/Gtg)	-	Lys43Arg (aag/aGg)/-	2/RHES
L4.2.2.ETH	His445Tyr (cac/Tac) His445Arg (cac/cGc)	Ser315Thr (agc/aCc)	Met306Ile (atg/atA)	-	-/ p.Gly69Asp	1/RHES
L3.1.1/Delhi-CAS	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	−12C>T Met306Ile (atg/atA)	-	-/-	1/RHE
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Met306Ile (atg/atC)	Unknown mutation	-/-	1/RHEZ
L4.2.2.ETH	Ser450Leu (tcg/tTg)	Ser315Thr (agc/aCc)	Met306Ile (atg/atA) Gly406Ser (ggc/Agc)	Gln141Pro (cag/cCg)	Lys43Arg (aag/aGg)/-	1/RHEZS
Total number of Mtb sub-lineages						20

Notes: R: rifampicin (RIF), H: isoniazid (INH), E: ethambutol (EMB), Z: pyrazinamide (PZA), S: streptomycin (STR), #: number.

M. tuberculosis Drug Resistance Associated Mutations

Of the 24 RIF resistance SNPs, including the four double mutations, 54.2%, 12.5%, and 12.5% were associated with Ser450Leu (tcg/tTg), His445Arg (cac/cGc), and His445Tyr (cac/Tac) substitutions, respectively. Only *katG* mutation at codon 315 was associated with INH resistance. Of the 19 *katG* mutations, including two double SNPs at codon 315, Ser315Thr (agc/aCc) accounted for 89.5% (17/19), and the remaining 10.5% were for the Ser315Arg (agc/agA) substitution. Of the 20 drug-resistant sub-lineages, 15 developed EMB resistance via nine resistance conferring mutations. The dominant resistance-associated mutations in EMB were guanine with adenine (atg/atA) and guanine with cytosine (atg/atC) substitutions, both of which replaced methionine with isoleucine. Seven isolates from L4.2.2, were resistant to PZA in *pncA* gene. Mutations in two genes (*rpsL* and *gidB*) are responsible for STR resistance (Table 1 and Figure 2).

Collectively, out of 20 Mtb resistant isolates, 19 resistant isolates were identified from PTB, 11 (55%) were resistant to ≥4 first-line anti-TB drugs. L4.2.2.ETH were the leading DR clone. The resistance of L4.2.2.ETH for STR was mainly (8/9) mediated with low-level drug resistance conferring mutations, *gibB* (Table 1). Except STR resistance mutations, the prevalent mutations conferred high-level DR (Table 2).

Discussion

This study identified the leading drug-resistant sub-lineages and drug resistance associated SNPs. Furthermore, characterization of the mutations was performed. As such, this finding shows a relatively high proportion and degree of MDR in L4.2.2. ETH/SIT149/. Resistant isolates of L4.2.2/SIT149 have also been reported in Ethiopia,[22–24] Saudi Arabia[11] and Tanzania.[17] The other prevalent DR Mtb lineage in Ethiopia is the Delhi-CAS (L3). Despite the widespread prevalence of DR L4.2.2/SIT149 isolates, much attention has not been paid to further characterization of downstream preventive and programmatic measures. It has been noted that L4.2.2/SIT149 and most Delhi-CAS isolates have sympatric relationships with Ethiopians and are more likely to be MDR than allopatric genotypes.[29] The great southward human migration from Eurasia 3000 years before likely imported L4.2.2 into Ethiopia.[27,58] Similarly, South Asia was predicted to be the origin of all L3 strains, with multiple independent introductions into North and Horn Africa.[59] Taken together, the high prevalence of DR-TB due to Mtb sub-lineages with sympatric host associations indicates high rate of TB transmission taking place for long period of time.

Most of the MDR isolates from L4.2.2. ETH/SIT149 formed clusters. Unlike this report, Shea et al concluded that the clustering rate of DR-Mtb is relatively low compared to that of the susceptible strain because of the transmission fitness cost.[53] As explained by Comas et al, fitness cost among dominant mutations is rare and MDR isolates are as competent as susceptible isolates.[60] Hence, conclusions such as "MDR isolates are transmission deficient due to fitness cost" must be interpreted with caution.

In the present study, 11.6% of the patients had MDR-TB. According to a WHO report, the prevalence of MDR-TB in Ethiopia is 1.1% and 12% among new and previously treated cases, respectively.[5] As such, Ethiopia was excluded from the WHO high-MDR-TB burden country list. However, the observation of high level of drug resistance among geographically restricted isolates is a warning sign that needs reassessment and further survey. Our WGS data showed that MDR isolates had extensive mutations, including double mutations. Such high-level mutations in multiple anti-TB drugs are proxy indicators of the possible occurrence of extensively drug-resistant TB in the near future.

Ser450Leu (tcg/tTg), Ser315Thr (agc/aCc), Met306Ile (atg/atA), and p.Gly69AspR were the dominant amino acids/SNPs substitutions responsible for resistance to RIF, INH, EMB, and STR, respectively. Based on the information in Table 2, except STR resistance determining mutation, *gidB* (p.Gly69AspR), all other prevalent mutations in this study confer high-level drug resistance. Dominant mutations have no fitness cost owing to compensatory mutations and confer high-level drug resistance.[60] The characteristics and clinical interpretations of some of the prevalent mutations are presented below (Table 2).

High-level drug resistance is a complex evolutionary process that results in TB hotspot regions and inappropriate drug treatment settings.[61] As described in Table 1 and Table 2, DR-Mtb strains develop high-level resistance to multiple first-line anti-TB drugs. Additionally, as shown in Figure 1, the DR strains contain both clustered and distinct isolates. This

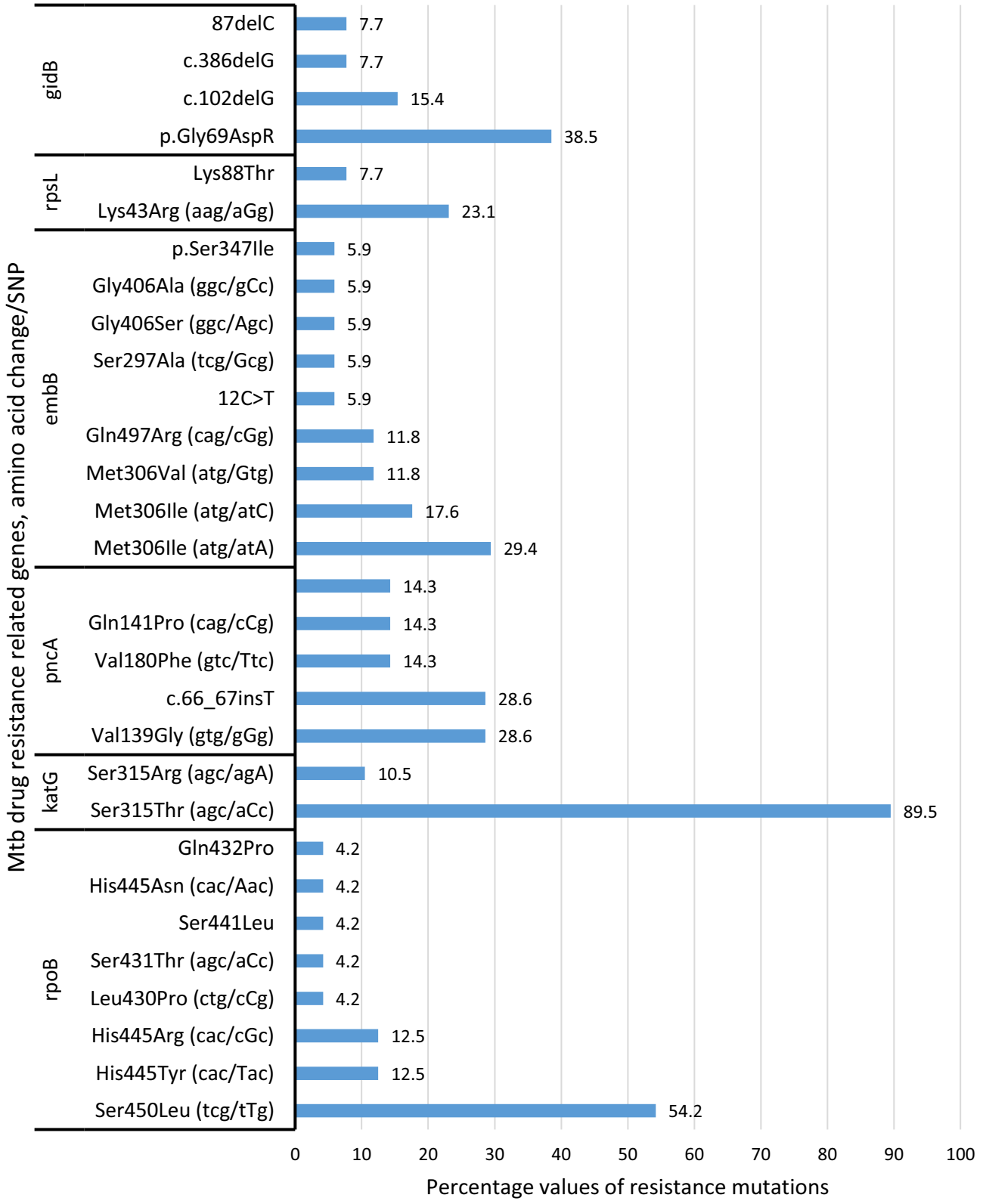

Figure 2 Genes, amino acid substitutions (single nucleotide polymorphism) associated with first line anti-TB drugs, Northwest Ethiopia, 2023.
Notes: The RIF resistance was determined with *rpoB* gene with eight types of amino acid (aa) substitutions. INH resistance was mediated with katG gene with two aa substitutions. The PZA resistance was conferred with *pncA* gene with mutations at four sites. Nine mutations at *embB* gene were associated with EMB resistance. The STR resistance was mediated with two genes, *rpsL* and *gidB*. The nucleotide substitutions are indicated within the bracket where the capitalized letter is the substituted nucleotide.

Table 2 Describe the Level of Resistance Associated with Each Resistance Marker, Northwest Ethiopia, 2023

Genes and Mutations		Clinical Interpretation	References
rpoB	Ser450Leu (tcg/tTg)	Confer high levels of RIF resistance More common among MDR-TB strains Have compensatory mutations in rpoC	[53,54]
	His445Tyr (cac/Tac)	More common among RIF mono resistance Had compensatory mechanism	[54]
	His445Arg (cac/cGc)	Have compensatory mechanism Confer high levels of RIF resistance	[54]
	Leu430Pro (ctg/cCg)	Confer low level of resistance	[53]
	Ser431Thr (agc/aCc)		
	Ser441Leu		
	His445Asn (cac/Aac)		
	Gln432Pro		
katG	Ser315Thr (agc/aCc)	Confer a high level of resistance Has no fitness cost, hence prevalent among MDR-TB *ahpC* is compensatory mutation	[55]
	Ser315Arg (agc/agA)		
pncA	Val139Gly (gtg/gGg)		
	c.66_67insT		
	Val180Phe (gtc/Ttc)		
	Gln141Pro (cag/cCg)		
embB	Met306Ile (atg/atA)	More prevalent among MDR-TB The embB306 locus considered as marker for rapid detection of MDR and XDR-TB	[56]
	Met306Ile (atg/atC)		
	Met306Val (atg/Gtg)		
	Gln497Arg (cag/cGg)		
	12C>T		
	Ser297Ala (tcg/Gcg)		
	Gly406Ser (ggc/Agc)		
	Gly406Ala (ggc/gCc)		
	p.Ser347Ile		
rpsL	Lys43Arg (aag/aGg)	Confer high level STR-resistance	[57]
	Lys88Thr		
gidB	p.Gly69AspR	Confer low-level STR resistance No fitness cost is associated with this mutation	
	c.102delG		
	c.386delG		
	87delC		

indicated that the epidemics of DR in the study area is driven by both independent evolution and transmission of acquired resistance. This again happens in countries where the implementation and adherence to WHO recommended TB control strategy is ineffective. Lower disease severity, low inflammation, high secondary case rate and high rate of MDR occurred among sympatric host pathogen association.[29,62,63]

The fitness of drug-resistant strains is heterogeneous.[64] For instance, the fitness of DR Mtb strains showed comparable fitness with drug susceptible strains in some phylogeographic regions such as former Soviet Union.[64] DR Mtb strains in this region might regain their pre-resistance fitness through compensatory evolution which again happen in areas where TB transmission takes place for long period of time.[64]

Limitation of the Study

Most of the drug-resistant sub-lineages were obtained from Mtb isolates archived in the laboratory. Hence, sampling bias is likely. Furthermore, the sample size was too small to obtain strong epidemiological information. Therefore, caution must be taken when interpreting the results.

Conclusion and Recommendations

Nineteen of the 20 drug-resistant isolates were obtained from PTB and L4.2.2. ETH/SIT149 was the dominant drug-resistant isolate followed by Delhi-CAS/L3. Furthermore, most of L4.2.2.ETH/SIT149 isolates were also resistant to all first-line anti-TB drugs, including STR. Proportionally, 56.5%, 89.5%, and 47% of RIF, INH, and EMB resistance-associated mutations were Ser450Leu/(tcg/tTg), Ser315Thr/(agc/aCc), and Met306Ile/(atg/atA(C)) substitutions, respectively. These mutations confer high levels of resistance. Mtb isolates that are resistant to all first-line anti-TB drugs, including STR, must be considered as a national threat. The high prevalence of drug resistance among geographically restricted sub-lineages indicates the ongoing local TB epidemics and uncontrolled TB transmission. Hence, active surveillance of DR-TB must be strengthened. Further evolutionary characterization of MDR isolates is very desirable to identify evolutionary forces driving the MDR-TB pandemic.

Data Sharing Statement

The WGS data are deposited in GenBank public database with bio project ID: PRJNA975069 and will be freely available shortly after publication of the manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Research and Ethical Review Committee of Science College of Bahir Dar University (reference number PGRCSV/111/2012). Written informed consent was obtained from each participant prior to data collection. This study was conducted in accordance with the principles of the Declaration of Helsinki. All information obtained from the study participants was coded to maintain confidentiality.

Acknowledgments

The authors would like to express our gratitude to the Institute of Biotechnology and the Institute of Carlos III for their administrative support. Appreciation also goes to the International Federation for Clinical Chemistry (IFCC) for providing financial support to Daniel Mekonnen through the IFCC Professional Exchange Program (PEP). We also thank the Amhara Public Health Institute Mycobacteriology Laboratory staff, Felege Hiwote Referral Hospital DOTS clinic physicians, and the Pathology laboratory Team. DOTS clinic staff of Han Health, Bahir Dar, and Shum-Abo Health Centers also deserve special thanks for their support in the data collection. We also thank the Institute of Carlos III Mycobacteriology Laboratory staff, Martha and Rackel, for their support during the DNA extraction and WGS.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Sample collection was funded by the Institute of Biotechnology, Bahir Dar University, through the Endalkachew Nibret Mega Project. The Mtb culture and identification-related laboratory supply was supported by the Amhara Public Health Institute, Bahir Dar, Ethiopia. Whole-genome sequencing was performed with the great support of National Center of Microbiology, Institute of Carlos III, Madrid, Spain. The International Federation for Clinical Chemistry (IFCC) provided financial support to Daniel Mekonnen through the IFCC Professional Exchange Program (PEP) for three months stay in Madrid Spain for conducting the WGS analysis.

Disclosure

None of the authors has any relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending and royalties.

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