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Multidrug-resistant and heteroresistant *Mycobacterium tuberculosis* and associated gene mutations in Ethiopia

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SUMMARY

Background: The prevalence of multidrug-resistant tuberculosis (TB) among new and retreatment cases in 2011 in Ethiopia was 2.7% and 17.9%, respectively. However, data on heteroresistance and gene mutation profiles of *Mycobacterium tuberculosis* were not documented.
Methods: A cross-sectional study was conducted on 413 TB-positive clinical specimens submitted between 2012 and 2014 to Bahir Dar Regional Laboratory Center for confirmation of multidrug resistance. Resistance determining genes were analyzed using a line probe assay.
Results: Of 413 *M. tuberculosis* isolates, 150 (36.3%) were multidrug-resistant, 19 (4.6%) were resistant only to rifampicin, and 26 (6.3%) were resistant to isoniazid. Of 169 rifampicin-resistant and 176 isoniazid-resistant isolates, only eight (4.7%) showed rifampicin heteroresistance and only two (1.13%) showed isoniazid heteroresistance. Failing of the *rpoB* WT8 gene with corresponding hybridization of *rpoB* MUT3 (S531L substitution) accounted for 85 (50.3%) rifampicin-resistant mutations. Among 176 isoniazid-resistant isolates, 155 (88.1%) strains had the Ser315Thr1 substitution.
Conclusions: The prevalence of multidrug-resistant *M. tuberculosis* was high in the study area. Ser531Leu and Ser315Thr1 substitutions were the highest gene mutations for rifampicin and isoniazid, respectively.

1. Introduction

Multidrug-resistant tuberculosis (MDR-TB) is caused by strains of *Mycobacterium tuberculosis* that are resistant to isoniazid (INH) and rifampicin (RMP).[1,2] Despite the availability of highly efficacious treatment for decades, tuberculosis (TB) remains a major global health problem.[1,3–5] Globally, 3.5% of MDR-TB has been reported in new TB cases and 20.5% in previously treated TB cases.[6,7] In Ethiopia in 2011, the prevalence of MDR-TB among new and retreatment cases was reported to be 2.7% and 17.9%, respectively.[8]

An erratic drug supply, suboptimal physician prescription, poor patient adherence,[9] initial high bacterial population, and monotherapy have been associated with the emergence of resistance.[10] Some patients with TB harbor mixed populations of drug-susceptible and resistant organisms, a phenomenon that is referred to as heteroresistance. Therefore, heteroresistant strains are precursors for full resistance.[11]

The genetic background of *M. tuberculosis* related to INH resistance is complex. However, mutations in several genes, including *katG* (catalase peroxidase coding genes),[12] *ahpC*, *inhA*, *kasA*, and *ndh*, have all been associated with INH resistance.[10,13,14] Between 50% and 95% of INH-resistant strains contain mutations in codon 315 of the *katG* gene.[9] Furthermore, 20% and 35% of INH-resistant strains contain mutations in the *inhA* regulatory region.[13,14] The most common *inhA* mutation occurs in its promoter region (C15T) and this is frequently associated with monoresistance.[13,14] Strains bearing mutations in the coding region of *inhA* show low-level resistance.[10,15]

Mutations in the RNA polymerase β subunit (*rpoB*) gene have been found in about 96% of RMP-resistant *M. tuberculosis*

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isolates.[14–16] Mutations in codons 531 and 526 are the most frequently reported mutations.[14–16]

Information on the current prevalence of MDR-TB, heteroresistance, and drug resistance mutations has not been documented in Amhara National Regional State (ANRS), Ethiopia. This study was conducted to determine the prevalence of MDR-TB, heteroresistance, and gene mutations to RMP and INH among presumptive MDR-TB cases in ANRS, Ethiopia.

2. Materials and methods

2.1. Study design and sampling technique

A cross-sectional study was conducted between May 2012 and February 2014. During the study period, 856 presumptive MDR-TB cases (sputum and extrapulmonary (peritoneal fluid, tissue, lymph node aspirate, and pus specimens)) were referred to the Bahir Dar Regional Health Research Laboratory Center (BRHRLC). However, only 413 (48.2%) of these clinical samples were TB-positive. This study included the 413 *M. tuberculosis* isolates for gene mutation analysis.

2.2. Specimen processing

The clinical samples were processed using the *N*-acetyl-L-cysteine NaOH (NALC-NaOH) method. The processed samples were suspended in 1.0 ml sterile phosphate buffer (pH 7.0) and then 100 μl of resuspended pellet was inoculated onto two Lowenstein–Jensen (LJ) medium slants. Smears for microscopic examination were stained using the Ziehl–Neelsen (ZN) method.

DNA was extracted from samples that were smear- and/or culture-positive using GenoLyse chemical methods. From the extracted DNA, 5 μl was used directly for PCR amplification. Master mix preparation, DNA addition, amplification, hybridization, and interpretation were performed as recommended by the manufacturer (Hain Lifescience GmbH, Nehren, Germany).[13,17] Resistance determining genes were analyzed using a line probe assay (LPA).

2.3. LPA interpretation

Susceptibility to anti-TB drugs was defined as hybridization (presence of a band) to all the wild-type (WT) probes and no hybridization (absence of a band) to the mutant probes. The absence of hybridization of any WT and/or hybridization of any mutant gene indicates resistance to the respective drugs. Hybridization of WT and mutant genes indicates heteroresistance or a mixed infection (Figure 1).

2.4. Statistical analysis

All data were entered, cleared, and analyzed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp. Armonk, NY, USA). Descriptive statistics were used to visualize differences within the data. Binary logistic regression was used to assess possible factors associated with MDR-TB and heteroresistance. Gene mutations were analyzed manually.

2.5. Data quality assurance

DNA extraction positive (H37Rv) and negative controls and master mix controls were used. Fifty isolates were characterized at the national TB laboratory using the BACTEC MGIT (Mycobacteria Growth Indicator Tube) 960 TB system (BD Diagnostics, USA). Lot-to lot quality assurance systems were in place to verify the quality of the commercial kit. All procedures were done using standard operating procedures.

2.6. Ethical considerations

Ethical clearance was obtained from the Amhara Regional Health Bureau Research Ethics Review Committee and official permission was obtained from BRHRLC.

Figure 1. Line probe assay strips showing controls and rifampicin (RMP) and isoniazid (INH) resistance-associated banding patterns; Ethiopia, 2015.

Table 1
Demographic characteristics and profile of presumptive MDR-TB cases ($N = 413$); Ethiopia, 2015

Variables		Number	Percentage
Sex	Male	229	55.4
	Female	184	44.6
Age, years	≤18	22	5.3
	19–34	211	51.1
	35–45	97	23.5
	>45	83	20.1
TB history	New cases	71	17.2
	Retreatment cases	342	82.8
Treatment history	New	71	17.2
	Relapse	160	38.7
	Failure	132	32
	Defaulter	32	7.7
	Other	18	4.4
Total	413	100	

MDR, multidrug-resistant; TB, tuberculosis.

3. Results

3.1. Multidrug-resistant tuberculosis

A total of 413 TB-positive patients were included in the study. Of these, 229 (55.4%) were males. The mean age of participants was 34.5 years and 211 (51.1%) were in the 19–34 years age group. Moreover, 342 (82.8%) patients were retreatment TB cases and 71 (17.2%) were newly identified TB cases. With regard to their TB history, 160 (38.7%) were relapse cases, 132 (32%) were failure cases, and 32 (7.7%) were defaulters (Table 1).

Of the 413 *M. tuberculosis* cases, 150 (36.3%) were MDR-TB (RMP+INH), 19 (4.6%) were resistant only to RMP, and 26 (6.3%) were resistant to INH.

Considering possible associated variables such as gender, age, type of TB, and history of TB treatment, none was significantly associated with MDR-TB or with RMP or INH resistance (data not shown).

3.2. Heteroresistance

Of 169 RMP-resistant and 176 INH-resistant *M. tuberculosis* isolates, eight (4.7%) showed RMP heteroresistance and two (1.13%) showed INH heteroresistance. The possible variables such as gender, age, type of TB, and history of TB treatment were not significantly associated with heteroresistance (data not shown).

3.3. RMP and INH resistance-associated gene mutations

Of the 413 isolates, 169 (40.9%) were resistant to RMP. Failing of the *rpoB* WT8 gene with corresponding hybridization of *rpoB* MUT3 (Ser531Leu substitution) accounted for 85 (50.3%) mutations. Moreover, failing of WT7 with the appearance of mutants 2A and 2B (H526Y and H526D substitutions, respectively) shared 14.8% of RMP-resistant gene mutation. There was no failing of the *rpoB* WT1, 5, or 6 genes and no hybridization of the *rpoB* MUT1 probe (Table 2).

Of 176 INH-resistant strains, 155 (88.1%) were due to failing of the *katG* WT gene with hybridization of the *katG* MUT probe (Ser315Thr1 substitution). Of the rest, five (2.8%) were due to failing at the *inhA* WT1 gene (C15T substitution). Moreover, three

Table 2
Frequency and pattern of *rpoB*, *katG*, and *inhA* mutations of *Mycobacterium tuberculosis*; Ethiopia, 2015

rpoB gene			*katG* gene			*inhA* gene			*n* (%)
Failing WT probe	Mutation	Location of codons of WT/mutant/gene	Failing WT probe	Mutation	Location of codons of WT/mutant/gene	Failing WT probe	Mutation	Analyzed nucleic acid position	
MDR ($n = 150$)									
WT2 and 3	No	510–513	WT	MUT1	S315T1	No	No	No	9 (6)
WT2, 3, and 4	Unknown	510–520	WT	MUT1	S315T1	No	No	No	3 (2)
WT3 and 4	Unknown	513–519	WT	MUT1	S315T1	No	No	No	20 (13.3)
WT3	Unknown	514–515	WT	MUT1	S315T1	No	No	No	1 (0.67)
WT2	No	511–512	No	No	No	WT1	MUT1	C15T	1 (0.67)
WT7	MUT2A	H526Y	WT	MUT1	S315T1	No	No	No	14 (9.3)[a]
WT7	MUT2B	H526D	WT	MUT1	S315T1	No	No	No	5 (3.3)[b]
WT7	Unknown	526	WT	MUT1	S315T1	No	No	No	14 (9.3)
WT8	MUT3	S531L	WT	MUT1	S315T1	No	No	No	60 (40)[c]
WT8	Unknown	531–533	WT	MUT1	S315T1	No	No	No	6 (4)
WT8	MUT3	S531L	WT	No	Dele, 315	No	No	No	11 (7.3)[a]
WT8	MUT3	S531L	No	No	No	WT1	MUT1	C15T	1 (0.67)
WT8	MUT3	S531L	WT	MUT1	S315T1	WT1	MUT1	C15T	3 (2)[b]
WT8	MUT3	S531L	WT	MUT2	S315T2	No	No	No	1 (0.67)
WT8	Unknown	531–533	WT	No	Dele, 315	No	No	No	1 (0.67)
RMP-MR ($n = 19$)									
WT7	MUT2A	H526Y	No	No	No	No	No	No	4 (21)
WT7	MUT2B	H526D	No	No	No	No	No	No	2 (10.5)
No	MUT2A	H526Y	No	No	No	No	No	No	1 (5.3)[a]
WT8	MUT3	S531L	No	No	No	No	No	No	9 (47.4)
WT8	Unknown	531–533	No	No	No	No	No	No	3 (15.8)
INH-MR ($n = 26$)									
No	No	No	WT	MUT1	S315T1	No	No	No	19 (73.1)
No	No	No	WT	No	Dele, 315	No	No	No	4 (15.4)
No	No	No	No	No	No	WT1	MUT1	C15T	3 (11.5)

C, cysteine; D, aspartate; Dele, Deletion; H, histidine; INH, isoniazid; L, leucine; LPA, line probe assay; MDR, multidrug-resistant; MR, Monoresistant; MUT, mutant; RMP, rifampicin; S, serine; T, threonine; WT, wild-type; Y, tyrosine.
No = no failing of the WT gene, or no appearance of mutant gene; Unknown = there were two or more mutant genes but the LPA did not have a probe for these mutant genes.
[a] One patient had RMP heteroresistance.
[b] One patient had INH heteroresistance.
[c] Five patients had RMP heteroresistance.

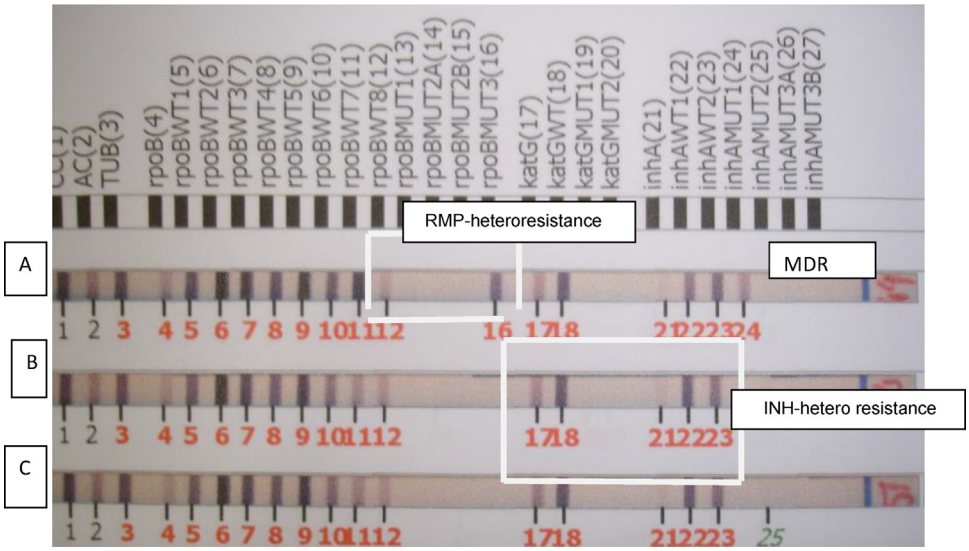

Figure 2. Line probe assay strips showing the heteroresistant population of *Mycobacterium tuberculosis*; Ethiopia, 2015.
Source: Barnard M, Parsons L, Miotto P, Cirillo D, Feldmann K, Gutierrez C, Somoskovi A. Molecular detection of drug-resistant tuberculosis by line probe assay. Laboratory manual for resource-limited settings (http://www.finddiagnostics.org).

(1.7%) INH resistance cases were due to mutations from both Ser315Thr1 and C15T substitutions (Table 2).

Based on gene mutation analysis, 60 (40%) MDR cases were due to Ser531Leu:Ser315Thr1 substitution. Moreover, the combination mutation of failing at WT3, 4 of RMP and Ser315Thr1 substitution of INH accounted for 20 (13.3%) MDR-TB. H526Y and Ser315Thr1 substitutions were MDR-TB mutations in 14 (9.3%) (Table 2). Out of 26 cases of RMP monoresistance, nine (47.4%) were due to Ser531Leu substitution and four (21%) to H526Y substitution. Nineteen (73.1%) of the INH monoresistance cases were due to Ser315Thr1 substitution and three (11.5%) to C15T substitution (Table 2).

4. Discussion

4.1. Multidrug-resistant tuberculosis

This study showed that MDR-TB is a serious public health problem in Ethiopia. A high prevalence of MDR-TB with lower heteroresistance might indicate a high transmission rate of primary resistant TB in this area.

The prevalence of MDR-TB in this study was higher than the drug resistance survey in Ethiopia.[8] The reason for this might be differences in study population; the present study population comprised presumptive MDR-TB cases, but the Ethiopia drug resistance survey involved smear-positive TB cases. Furthermore, the prevalence is higher than those reported from Germany (4%), Iran (12.2%), China (5.6%), in World Health Organization (WHO) reports, and Swaziland.[7,18–22] The reason for this might again be differences in study population, but might also be related to the time of the study, geography, and methodological differences.

4.2. Heteroresistance

The prevalence of RMP heteroresistance (mixed infection) was 1.9% ($n = 8$). Only two (0.5%) cases of INH heteroresistance were detected in this study. The proportion of heteroresistance in this study is lower than that reported in studies conducted in Uzbekistan and India.[23,24] This discrepancy might be the results of several factors. For instance, the prevalence of TB and MDR-TB might influence the occurrence of heteroresistance, like in India.[25] A high TB incidence certainly increases the risk of superinfection. Furthermore, differences in the source of isolates (direct specimen, culture) and typing methods has resulted in differences in detecting heteroresistance.[25]

These heteroresistant populations of bacteria might occur during a chronic infection, because several subpopulations may coexist in the same patient with different drug susceptibility profiles.[22] Heteroresistance may develop during treatment or by mixed/superinfection with sensitive and resistant strains.[26] The LPA is capable of detecting the presence of heteroresistance since the strips contain both WT and mutant probes (Figure 2A: bands 12 and 16).

4.3. RMP and INH resistance-associated gene mutations

The assessment of gene mutations showed that codon 531 of the *rpoB* gene and codon 315 of the *katG* gene accounted for 50.3% and 88.1% of RMP and INH resistance, respectively. This finding is in agreement with those of studies done in China, Sweden, Turkey, Ethiopia, and India.[15,27–30] However, the level of Ser531Leu substitution was lower than reported in studies done in Brazil, Pakistan, China, and Nepal.[31–34] This might be due to differences in the strain and lineage of isolates. The mutation frequency of INH resistance due to C15T substitution in this study was 1.9% ($n = 8$); however, studies done in Pakistan, China, and Nepal have shown a higher prevalence of C15T substitution.[32–34]This study showed a high prevalence of MDR-TB and a lower prevalence of hetero-resistant *M. tuberculosis*. Ser531Leu and Ser315Thr1 substitutions were the highest gene mutations for rifampicin and isoniazid, respectively.

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Conflict of interest: We the authors declare that we have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.06.013.

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