

Prevalence and Distribution of Multidrug-Resistant Mutations in *Mycobacterium tuberculosis* in Tanzania

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ABSTRACT

Background: Molecular identification of mutations resulting in multidrug-resistant tuberculosis (MDR-TB) is an important approach for improving understanding of MDR-TB epidemiology and planning for appropriate interventions. We aimed to estimate the prevalence and distribution of mutations causing MDR-TB as well as determine the gene distribution among patients previously treated for TB.

Methods: This was a cross-sectional, hospital-based study conducted from April 2017 to October 2018 at Kibong'oto Infectious Diseases Hospital (KIDH). KIDH is the national MDR-TB referral hospital. Participants were patients presumptively diagnosed with MDR-TB and referred to KIDH from district and regional hospitals across Tanzania. Sputum samples were collected and analysed using the Xpert MTB/RIF assay, direct sputum smear fluorescence microscopy, culture on Lowenstein-Jensen medium, and line probe assay using the GenoType MTBDRplus VER 2.0 system. Demographic information and mutation frequencies were reported as counts and percentages and analysed using descriptive statistics.

Results: A total of 208 (69.3%) participants had *rpoB* gene mutations conferring resistance to only rifampicin; 92 (30.7%) had *rpoB*, *katG*, and *inhA* mutations conferring resistance to rifampicin and isoniazid; 78 (26%) had *rpoB* and *katG* mutations conferring resistance to rifampicin and isoniazid; and 14 (4.7%) had *rpoB* and *inhA* mutations conferring resistance to rifampicin and isoniazid.

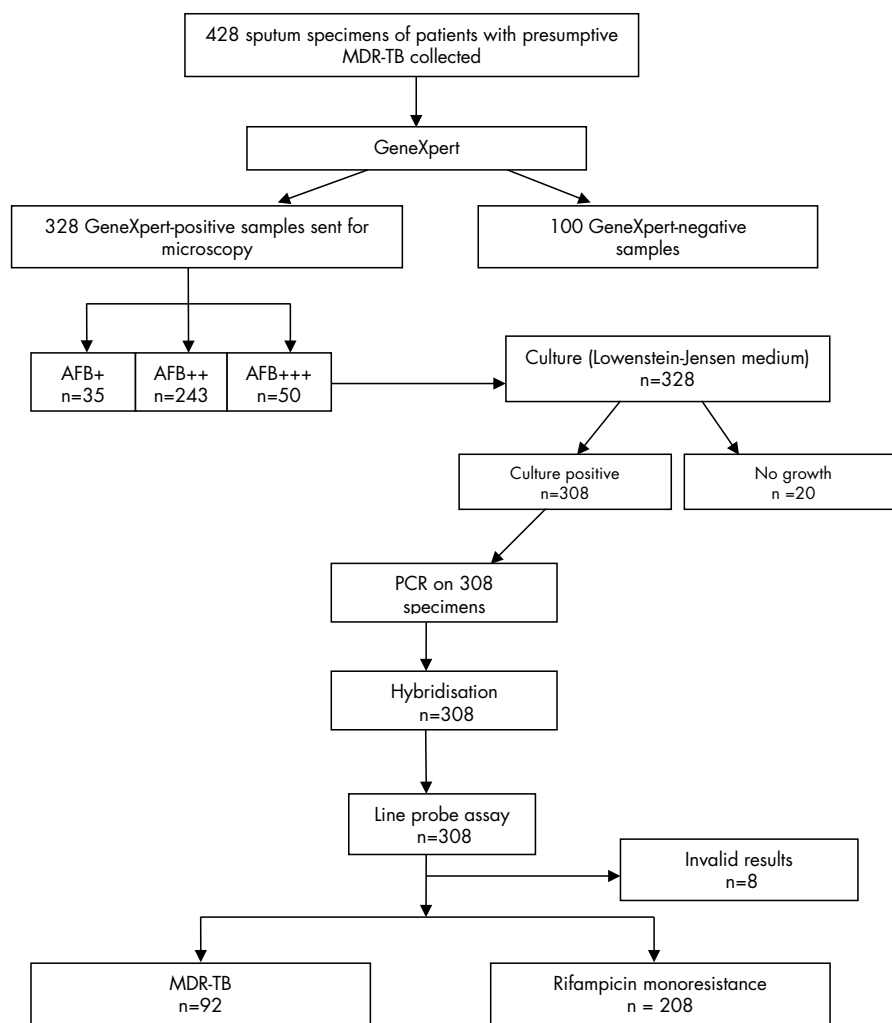
Conclusion: The mutation prevalences identified in this study indicate the most frequent mutations were the S531L mutation of the *rpoB* gene, the S315T1 mutation of the *katG* gene, and the S315T mutation in the promoter region of the *inhA* gene. To control the emergence and spread of MDR-TB, drug sensitivity testing must be carried for GeneXpert-confirmed TB patients prior to initiating second-line anti-TB regimens.

INTRODUCTION

Tuberculosis (TB) continues to be a disease of public health importance worldwide. The emergence of multidrug-resistant TB (MDR-TB) is a serious challenge to TB control.¹ MDR-TB, defined as resistance to both isoniazid and rifampicin, is a growing public health problem in resource-poor regions where adequate diagnosis and treatment are often unavailable.²⁻⁵ The World Health Organization (WHO) report of 2018 estimates that there were 558,000 new cases of rifampicin-resistant TB in 2017, 82% of which were MDR-TB.⁶ MDR-TB is estimated to affect 3.5% of patients diagnosed with TB for the first time and 20.5% of those previously treated for TB. WHO estimates that about 5% of all TB patients progress to MDR-TB, which had a peak mortal-

ity rate of more than 40% in 2013.^{7,8} Tanzania is among the 30 countries most affected by TB and MDR-TB, with an MDR-TB prevalence of 1.1% in 2010.^{6,9,10}

Resistance to anti-TB agents by *Mycobacterium tuberculosis* is caused by mutations in genes or the promoter regions of genes involved in drug activation or encoding drug targets. Molecular mechanisms for resistance to rifampicin are known to involve chromosomal mutations in the RNA polymerase β -subunit gene (*rpoB*), which encodes the β subunit of the *M. tuberculosis* RNA polymerase chain.¹¹⁻¹⁴ Isoniazid is one of the most effective anti-TB drugs. The majority of the mutations responsible for high-level *M. tuberculosis* isoniazid resistance are found in the catalase peroxidase gene (*katG*).¹⁵ Isoniazid resistance also commonly results from mutations in the

FIGURE 1. Sample Collection and Processing Flow Chart

Abbreviations: AFB, acid-fast bacilli; MDR-TB, multidrug-resistant tuberculosis; PCR, polymerase chain reaction

promoter region of the enoyl acyl carrier protein reductase gene (*inhA*)¹⁵; these mutations increase *inhA* expression and confer low-level resistance to isoniazid.¹⁶ The lower susceptibility to isoniazid is associated with mutations in the structural region of *inhA*, which lower affinity to drug-NAD adducts.¹⁶

The molecular detection of *M. tuberculosis* mutations is important for the understanding of TB epidemiology, as it can help predict transmission rates and identify dominant strains, strains with enhanced capacity to spread, and strains associated with outbreaks¹⁷ and severe disease.^{18,19} Effective genotypic monitoring of the emergence of drug-resistant strains of *M. tuberculosis* is pivotal to TB control, more so than

the detection of drug resistance by phenotype, which suffers from protracted identification of resistant strains. There is accumulating evidence correlating gene mutations with phenotypic resistance; however, the relevant data are sparse and inconsistent, particularly in sub-Saharan Africa where the disease burden is highest.¹⁸

This study aimed to estimate the prevalence and distribution of mutations causing MDR-TB as well as determine the gene distribution among patients previously treated for TB who presented at Kibong'oto Infectious Disease Hospital (KIDH) in Sanya Juu, Tanzania.

TABLE 1. Sociodemographic Characteristics (N=300)

Characteristics	n (%)
Age, years	
20-30	85 (28.3)
31-40	101 (33.7)
41-50	82 (27.3)
≥50	32 (10.6)
Gender	
Female	109 (36.3)
Male	191 (63.7)
Marital status	
Single	165 (55)
Married	135 (45)

METHODS

Design and Settings

This was a hospital-based cross-sectional study conducted from April 2017 to October 2018 at KIDH, a national referral hospital for patients from all parts of Tanzania. KIDH is currently the country's largest referral hospital for MDR-TB management, with a dedicated 40-bed capacity in addition to separate facilities for treating drug-susceptible TB.

Study Participants and Inclusion Criteria

We enrolled referred inpatients aged 18 years and older. A structured questionnaire was administered by interviewers to collect sociodemographic information in addition to that obtained from the hospital files. We excluded admitted patients on treatment for diseases other than TB and those coming from outside of Tanzania.

Sample and Data Collection

Sputum samples were collected from each participant in a sterile Falcon tube (BD Biosciences, Bedford, MA, USA). The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) for molecular detection of MDR-TB was used to test all sputum samples.^{20,21} Direct sputum smear fluorescence microscopy^{22,23} was used to test for acid-fast bacilli (AFB) for all enrolled

patients. All AFB-positive sputum samples were cultured on the Lowenstein-Jensen (LJ) medium (HiMedia Laboratories GmbH, Einhausen, Germany). Recovered *M. tuberculosis* colonies from LJ medium culture-positive sputum samples were used to perform a line probe assay (LiPA) employing the GenoType MTBDRplus VER 2.0 system (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions (Figure 1).²⁴

LiPA

M. tuberculosis DNA was extracted from recovered *M. tuberculosis* colonies using the GenoLyse DNA extraction kit (Hain Lifescience GmbH) followed by a polymerase chain reaction sequence to amplify the *rpoB* gene encoding the β -subunit of RNA polymerase, the *katG* gene encoding for catalase peroxidase, and the promoter region of the *inhA* gene encoding for NADH enoyl ACP reductase, for the detection of genomic mutations associated with MDR-TB. The primers and polymerase included in the MTBDRplus Ver 2.0 assay kits were used to amplify the genes, and the H37Rv quality control *M. tuberculosis* strain was used as the positive control. The *M. tuberculosis* mutations in the *rpoB* gene associated with rifampin resistance, and those in the *katG* and *inhA* genes associated with isoniazid resistance, were interpreted and determined by the band patterns on the LiPA strips after reverse hybridisation of the gene amplicates.²⁴⁻²⁶

Statistical Analysis

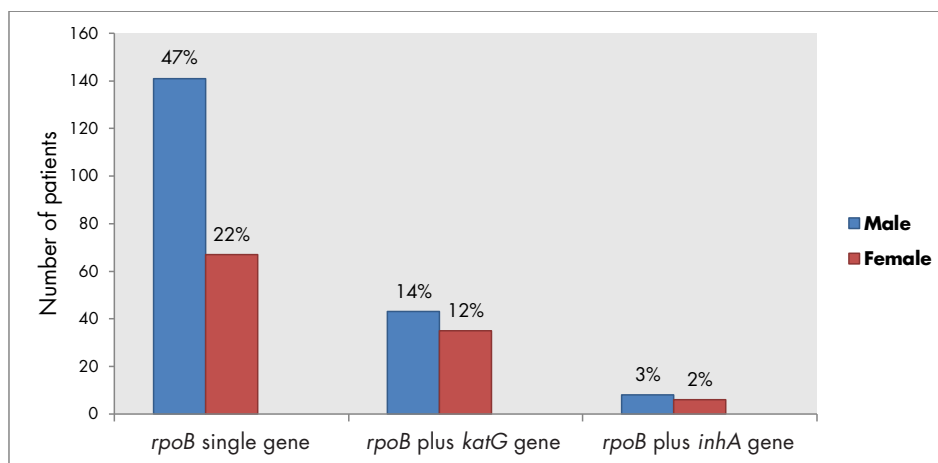
Data were analysed using IBM SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA). Data were summarised using frequency distributions and charts for categorical data and descriptive statistics (mean, median, standard deviation, and interquartile range) for numerical data. Chi-square tests were applied to assess patient sociodemographic and clinical characteristics associated with drug resistance, and Fisher's exact was used to calculate *P* values when comparing small frequencies (less than 5). The mutations rates in the *rpoB*, *katG*, and *inhA* genes, in relation to MDR-TB-negative and MDR-TB-positive status, were also estimated.

Ethical Considerations

Ethical approval was obtained from the Kilimanjaro Christian Medical University College Research and Ethics Committee (Certificate number 2039, April 2017). Permission from KIDH management was obtained from the medical officer in charge. All participants consented to participate in the study voluntarily after the study was explained to them. The patients' confidentiality and privacy were strictly observed.

RESULTS

A total of 428 presumptive sputum specimens were collected from newly referred patients to KIDH. Of these, 100 tested negative for TB using Xpert MTB/RIF, and these patients were

FIGURE 2. Prevalence and Distribution of Mutations among *Mycobacterium tuberculosis* Isolates by Sex

excluded from the study and treated as per routine hospital guidelines. Sputum smear fluorescence microscopy was carried out on 328 samples, which were then cultured on LJ medium. Twenty of the samples were LJ medium culture-negative, and the patients who submitted these samples were excluded and treated as per routine hospital guidelines. DNA extraction – using GenoLyse kits according to manufacturer's instructions – was performed on 308 specimens that showed growth in AFB culture. The LiPA was performed for 308 specimens, and 8 specimens returned invalid LiPA results; the patients who submitted these 8 specimens were excluded from the analysis and treated as per standard-of-care guidelines.

Out of the 300 patients recruited, 191 (63.7%) were male, and 109 (36.3%) were female. The mean patient age was 37.5 ± 10 years. Patients between of 20 and 40 years old were most affected by MDR-TB (Table 1). There were 208 (69.3%) isolates that had mutations conferring resistance to only rifampicin (rifampicin-monoresistant) and 92 (30.7%) isolates that had mutations conferring resistance to both rifampicin and isoniazid (multidrug-resistant [MDR] isolates) (Figure 2). The S531L mutation was observed in 182 (60.7%) of the isolates. Seventy-eight (26.0%) were found to have the *katG* gene mutations. Fourteen (4.7%) isolates had solitary *inhA* gene mutations (Table 2).

Prevalence and Distribution of *M. tuberculosis* Mutations by Sex

Among the 208 LiPA-screened patients with single *rpoB* gene mutations, 141 (67.8%) were male. Among 78 (26%) patients whose isolates had both *rpoB* and *katG* gene mutations, 43 (55.1%) were male. Fourteen patients (4.7%) had *rpoB* and *inhA* gene mutations, 8 (57.1%) of whom were male.

Rifampicin Resistance–Associated Mutations

Rifampicin resistance–associated mutations involving the *rpoB* gene were the most frequently encountered mutations in this study, appearing in 208 (69.3%) isolates (Table 3). The S531L (TCG→TTG) *rpoB* mutation was observed in 182 (60.7%) isolates. The frequency of the S531L mutation in the *rpoB* gene was significantly higher among rifampicin-monoresistant isolates than among MDR isolates ($P < .01$). Mutations in the *rpoB* gene region encompassing codons 513 to 519 were significantly more common among non-MDR-TB isolates than among MDR-TB isolates ($P < .01$) (Table 3).

Isoniazid Resistance–Associated Mutations

The *katG* 315ACC mutation was the most common isoniazid–rifampicin multidrug resistance–associated mutation identified in this study. The S315T1 *katG* gene mutation – with a codon change of AGC→ACC – occurred in 78 (84.8%) of 92 MDR isolates detected. Mutations at the promoter region of the *inhA* gene were also detected. Overall, the 315ACC mutation was found in 14 (15.2%) of the 92 MDR isolates: 7 with the MUT1 S315T variant (TCG→TGG), 1 with the WT 315 variant, and 6 with the WT1 C15T variant (GGC→ACC) (Table 3).

DISCUSSION

The present study highlights the prevalence and distribution of MDR *M. tuberculosis* mutations in Tanzania. The majority patients were between 20 and 40 years old, with male patients predominating. The frequency of MDR-TB in this age group has substantial socioeconomic implications, as young adult males are an important component of the economically productive population. A high MDR-TB prevalence among young adults has an acute impact on the national economy. TB control strategies need to set specific targets for all age groups, but for this group in particular. Our findings are comparable

TABLE 2. Prevalence and Distribution of Gene Mutations in *Mycobacterium tuberculosis* by Patient Characteristics (N=300)

Characteristics	Non-Multidrug-Resistant (n=208)					Multidrug-Resistant (n=92)		
	<i>rpoB</i> (Monoresistance)					<i>katG</i> and <i>rpoB</i> (n=78)	<i>rpoB</i> and <i>inhA</i> (n=14)	
	MUT1, MUT2B, (S315T1), (H526D) n=5 (%) ^a	MUT3 (S531L) n=182 (%) ^b	WT3 (513-517) n=9 (%) ^c	WT4 (519-519) n=7 (%) ^d	WT7, WT8 n=5 (%) ^e	MUT1 n=78 (%) ^f	MUT1 n=7 (%) ^g	WT, WT1 n=7 (%) ^h
Age								
20-30	2 (40%)	51 (28.02%)	1 (11.2%)	2 (28.57%)	1 (20%)	24 (30.7%)	2 (28.57%)	2 (28.57%)
31-40	1 (20%)	58 (31.9%)	4 (44.4%)	2 (28.57%)	3 (60%)	28 (35.9%)	3 (42.86%)	2 (28.57%)
41-50	2 (40%)	53 (29.1%)	0 (0%)	3 (42.86%)	1 (20%)	18 (23.1%)	2 (28.57%)	3 (42.86%)
≥50	0 (0%)	20 (0.11%)	4 (44.4%)	0 (0%)	0 (0%)	8 (10.3%)	0 (0%)	0 (0%)
Gender								
Female	3 (60%)	58 (31.9%)	3 (33.3%)	3 (42.86%)	1 (20%)	35 (44.9%)	2 (28.7%)	4 (57.1%)
Male	2 (40%)	124 (68.1%)	6 (66.7%)	4 (57.14%)	4 (80%)	43 (55.1%)	5 (71.3%)	3 (42.9%)

^{a-h}For each entry in the respective columns, the denominators are 5, 182, 9, 7, 5, 78, 7, and 7, respectively, which are counts for every mutation reported in a column across participant age groups and genders

with observations from previous studies in India, South Africa, and Zimbabwe, which revealed higher rates of MDR-TB among youths and young adults.²⁷⁻²⁹

We found a higher prevalence of MDR-TB among men in our study. Although the explanations regarding differences in immunity between men and women are incomplete, it is generally accepted that infectious diseases rarely affect males and females equally.³⁰ Females exhibit a more robust immune response to antigenic challenges, such as infection and vaccination, than males.³¹ This is mediated largely by sex hormones, the role of which – in TB – is supported by the fact that the male disadvantage does not arise until puberty.^{30,32} Sex hormones have diverse effects on many immune cell types, including B cells, T cells, neutrophils, dendritic cells, macrophages, and natural killer cells.³⁰ Other reasons for these gender differences may be related behavioural and exposure differences – including regarding social roles and risk behaviours, such as alcohol and tobacco consumption –

between the sexes, which make males more likely to acquire TB.^{30,33-35} In this regard, our findings are contradictory to what was previously reported in Pakistan and Afghanistan, where TB has been reported to be more prevalent among women than men³⁶; however, our results are comparable with other studies done in other parts of the world.^{30,37-42} It has been reported that nearly twice as many men as women have been diagnosed with TB globally.^{43,44} These findings are relevant for planning different TB control strategies and programmes, especially in low-resource settings.

We found a high prevalence of rifampicin monoresistance determined by *rpoB* gene mutations. Drug resistance is multifactorial and – in the presence of HIV infection, for example – higher rates anti-TB drug resistance could be attributable to HIV-associated malabsorption, mismanagement of TB cases, adherence challenges and antiretroviral and anti-TB drug interaction.⁴⁵⁻⁴⁹ Rifampicin is the most vital drug for TB treatment; therefore, resistance to rifampicin

TABLE 3. Prevalence and Distribution of Mutations, Amino Acid Changes, and Nucleotide Change in *Mycobacterium tuberculosis* Isolates (N=300)

Gene	Band Missing (WT#)/ Mutation Present (MUT#)	Mutation or Codons Involved	Amino Acid Change	Nucleotide Change	Non-MDR-TB n= 208	MDR-TB n=92	Fisher's Exact χ^2	P Value
<i>rpoB</i>								
	MUT1	S315T1	Ser→Thr	AGC→ACC	2	0		-
	MUT2B	H526D	His→Asp	CAC→GAC	3	0		-
	MUT3	S531L	Ser→Leu	TCG→TTG	182	0	191.48	<.01
	WT3	517-519	Asn→Lys	AAC→AAA	9	0	5.967	.02
	WT4	513-517	Lys→Phe Phe→His	AAA→TTC TTC→ATG	7	0	208.232	<.01
	WT7	526-529	His→Arg	CAC→CGC	3	0		-
	WT8	530-533	Leu533→Pro Ser531→Leu Ser531→Trp	CTG→CCG TTA→TTG (silent) TCG→TTG	2	0		-
<i>katG</i>								
	MUT1	S315T1	Ser→Thr	AGC→ACC	0	78	2229.57	<.01
<i>inhA</i>								
	MUT1	S315T	Ser→Thr	TCG→TGG	0	7		-
	WT	315			0	1		-
	WT1	C15T	Cys→Thr	GGC→ACC	0	6		-

has enormous implications for TB control programmes. Our findings are comparable with results from a study done in South Africa, which reported a rise in rifampicin monoresistance.^{45,50} A study conducted to evaluate anti-TB drug resistance surveillance in 19 countries reported the presence of rifampicin monoresistance in all of the countries.⁵¹ Rifampicin resistance has often been considered as a surrogate marker of MDR-TB, because it is highly correlated with concomitant isoniazid resistance.^{47,52} In this regard, all patients with rifampicin monoresistance ought to be treated as MDR-TB patients. Our findings, however, differed from study findings from Iran and Nigeria, which failed to detect rifampicin monoresistance.^{53,54}

We found a high prevalence of mutations in codon 315, with predominance of the ACC nucleotide sequence in the *katG* gene, which resulted in resistance to both rifampicin and isoniazid. This indicates that the amino acid at position 315 of *katG* is prone to mutation. These results correspond to what other researchers have reported, and it may be attributable to lifestyle factors, delays or difficulties in accessing health facilities, and patient immunocompromise or noncompliance to treatment.^{55,56} Mutations of the *katG* gene more strongly influence the development MDR-TB than mutations of the *inhA* gene.⁵⁷

We also observed the presence of *rpoB* and *inhA* gene mutations at codon 315 (S315T) and 15 (C15T) that resulted in resistance to both rifampicin and isoniazid. Our findings are comparable with those of other studies, whereby it has been reported that mutations at the *katG*-315, *rpoB*-531, and *inhA*-15 positions are associated with high rates of isoniazid-resistant TB.^{11,27} Furthermore, other studies have reported that mutations in codon 315 and the promoter region of the *inhA* gene are the most common and are associated with isoniazid resistance.^{14,55} These observations suggested that repeated administration of the same anti-TB drugs increases the risk of resistance, including multidrug resistance.

CONCLUSION

The most frequently detected mutations in our study were the S531L *rpoB* mutation, the S315T1 *katG* mutation, and the S315T mutation in the promoter region of the *inhA* gene. MDR-TB control strategies require an understanding of the evolution of these mutations. Further studies to evaluate these mutations in detail would increase our understanding of the epidemiology and transmission dynamics of drug-resistant *M. tuberculosis* in Tanzania to inform the planning, design, and implementation of innovative TB control strategies.

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