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Prevalence of mutations in genes associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates

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ABSTRACT

Purpose: To analyze prevalence of mutations in genes associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates from patients with possible MDR TB of Puducherry, South India and to explore the association of specific mutations conferring rifampicin (RIF) resistance.
Methods: We performed a commercial Genotype MDBDRplus V.2.0 assay for the rapid detection of rifampicin and isoniazid resistance directly on sputum specimens of patients with possible MDR TB.
Results: Totally 558 multidrug resistant, 293 RIF mono resistant and 923 INH mono resistant tuberculosis were detected from the 12,786 patients with possible MDR TB samples. The 50.5% mutations were observed in the region of S531L in MDR TB patients and 55.6% in rifampicin monoresistant cases. In total isoniazid monoresistant, 68.0% mutations were detected in *katG* gene, which is more prevalent in comparison to *inhA* gene 32.0%. There were about 57.9% and 32.2% MDR TB cases diagnosed in the age group of >15 to ≤ 45 years and >45 to ≤ 60 years respectively.
Conclusions: The rate of occurrences of mutations were found widely in the Rifampicin Resistant Determination Region (81 bp) of *rpoB* gene and the hypervariable region 530–533 codons of rpoB gene is alarming in the specification. The higher frequency of mutation in codons of *rpoB* (S531L) and *katG* (S315T) gene help to design simple, new and less expensive molecular techniques to use in peripheral laboratories.

Introduction

The emergence and spread of multi-drug resistant tuberculosis (MDR-TB) is menacing to global tuberculosis control. According to WHO, nearly 50% of the world's burden of MDR-TB cases is in India and China [33]. The prevalence of MDR-TB is increasing throughout the world both among new tuberculosis cases as well as among previously treated cases [34]. The World Health Organization has estimated that India accounted for 26% of the total number of TB cases worldwide in 2015, with 3.9% and 21% of the new and retreatment cases respectively being caused by multi drug resistant strains [16].

In the majority of drug-resistant *M. tuberculosis* clinical isolates, drug resistance is due to mutations in genes or promoters region of genes activating the drug or encoding the drug targets. Studies have pointed out that the *M. tuberculosis* becomes resistant to RIF due to the mutations in *rpoB*, INH due to *katG* and *inhA* [30]. Resistance to rifampicin in mycobacterium results from point mutations mainly located in the 507–533 region of the *rpoB* polypeptide [12,13,26]. The most common mutations observed in rifampicin resistant *M. tuberculosis* isolates are Ser531Leu, His526Asp or Tyr, and Asp516Val [37]. Rifampicin resistance in *M. tuberculosis* strains is conferred by a diverse group of mutations within a hypervariable region of the *rpoB* gene, which codes for a β-subunit of RNA polymerase [3,7]. More than 95% of rifampicin resistant isolates possess mutations within this hyper variable regions of the *rpoB* gene [10].Resistance to isoniazid is mostly associated with the amino acid substitution Ser315Thr in *katG* (in roughly 70% of INH-resistant strains) and the −15 C-to-T mutation in the *inhA* promoter (in 15–35% of INH-resistant strains) [8,18,25].

The rapid diagnosis of multidrug resistant tuberculosis patients, place them on treatment regimens is indispensable in controlling the MDR-TB in a community and limit the nosocomial spread of MDR-TB through proper infection control methods. The

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Table 1
MDR TB suspects on gender and age wise.

RNTCP criteria	Sex	Total no of MDR TB suspects received				Total MDR TB suspects received	Grand total
		≤15 years	>15 ≤ to 45 years	>45 to ≤60 years	> 60 years		
Failure	Male	2	251	164	51	468	557
	Female	1	71	13	4	89	
Re treatment case S+ at 4th month	Male	1	258	190	53	502	590
	Female	3	62	18	5	88	
Contact of known MDR TB case	Male	5	41	14	5	65	104
	Female	6	27	6	0	39	
S+ at diagnosis, re treatment case	Male	10	2600	1961	576	5147	6035
	Female	13	592	223	60	888	
Any follow up smear positive	Male	3	1125	836	247	2211	2702
	Female	7	326	120	38	491	
S - at diagnosis, re treatment case	Male	2	430	374	149	955	1266
	Female	5	186	92	28	311	
HIV TB case	Male	32	716	256	28	1032	1532
	Female	27	375	90	8	500	
Total	Male	55	5421	3795	1109	10380	12786
	Female	62	1639	562	143	2406	
Grand total		117	7060	4357	1252	12786	

World Health Organization (WHO) recommended Line-probe assays (LPAs), which can simultaneously identify the *M. tuberculosis* complex and detect genetic mutations in the *rpoB* gene region related to rifampicin resistance, *katG* and *inhA* gene regions related isoniazid resistance [24]. The main objective of this study the prevalence of mutations in genes associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates from patients with possible MDR TB of Puducherry, South India and to explore the association of specific mutations conferring rifampicin (RIF) resistance.

Materials and methods

Specimen collection and processing

The study was conducted retrospectively in the Intermediate Reference TB Laboratory at Government Hospital for Chest Diseases, Puducherry for a span of 42 months between July 2012 and December 2015. The two sputum samples were collected in 50 ml sterile falcon tubes for each patient and transported through cold chain mechanism from the nine districts (Villupuram, Tanjore, Nagapattinam, Thiruvarur, Cuddalore, Dindigul, Perambalore, and Trichy) of Tamil Nadu state in addition to Puducherry state as per the criterias of Revised National Tuberculosis Control Programme, India. Twelve thousands seven hundred and eight six (12,786) sputum samples were collected from various age groups for this study, which included ≤15 years (n-117), >15 to ≤45 years (n-7060), >45 to ≤60 years (n-4357) and >60 years (n-1252) as described in Table 1. The sputum samples received at Intermediate Reference TB Laboratory were assigned lab number and consecutively screened for acid fast bacilli (AFBs) using Fluorescence (FM) microscopy [32].The smear positive sputum samples in Fluorescence microscopy were directly processed by GenoType MTBDRplus V.2.0 assay (Hain Life Sciences). The smear negative samples were subjected to liquid culture using the BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) under stringent conditions. The culture positive samples from the MGIT system were in turn subjected to the GenoType MTBDRplus V.2.0 assay (Fig. 1). All the laboratory bench works related with potentially infectious specimens were performed in a Class II biosafety cabinet placed at Bio Safety Level III facility. All processed specimens were stored at −20 °C for the duration of the study to allow for re-testing of specimens giving discrepant results.

GenoType MTBDRplus V.2.0 assay

The GenoType MTBDRplus V.2.0 assay was performed according to the manufacturer's protocol ([15]). The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. All three steps were performed as per the WHO recommendations [29,39].

BACTEC MGIT 960 culture

The smear negative and discrepant samples were processed in MGIT 960 culture tube. A 500 µl sample was taken out from decontaminated sample and inoculated in BACTEC MGIT 960 tube. After the culture flashed positive, MGIT tubes were confirmed for acid fast bacilli by ZN staining and further subjected to confirm as *M. tuberculosis* complex using Capilia TB Neo (TAUNS Corporation, Japan) and checked for contamination by growth on blood agar medium for 48 h at 37 °C ([6,11,21]). The confirmed positive MGIT tube was processed with the Genotype MTBDRplus V.2.0 assay (Hain Life Science, Nehren, Germany) as per the manufacturer's protocol.

Results

The sputum samples were collected in 50 ml sterile falcon tubes from each person with possible MDR TB mainly based on the criteria of Revised National Tuberculosis Control Programme and totally 12,786 person's with possible MDR TB sputum samples from different age groups (Table 1) were processed for the Auromine O phenol staining. Among them, (83.8%) sputum samples were smear positive,(16.2%) samples were smear negative. The Conventional BACTEC MGIT 960 procedure was performed for all smear negative TB person's with possible MDR TB samples and no results/invalid obtained from Genotype MTBDRplus V.2.0 assay. The 2% contamination samples are received on request for the further processing by the Genotype MTBDRplus V.2.0 assay. In total, 1774 (13.87%) drug resistant strains were identified by Genotype MTBDRplus assay V.2.0; among them 558 were multidrug resistant, 293 were RIF mono resistant and 923 were INH mono resistant from high-risk patients. The number of MDR TB cases diagnosed from each criteria were tabulated as shown in Table 2.

Overall, 83.8% smear positive specimens gave interpretable results within 2–3 days by Genotype MTBDRplus V.2.0 assay and only 16.2% of the samples gave smear negative results by Fluorescence Microscopy and those samples are further established with

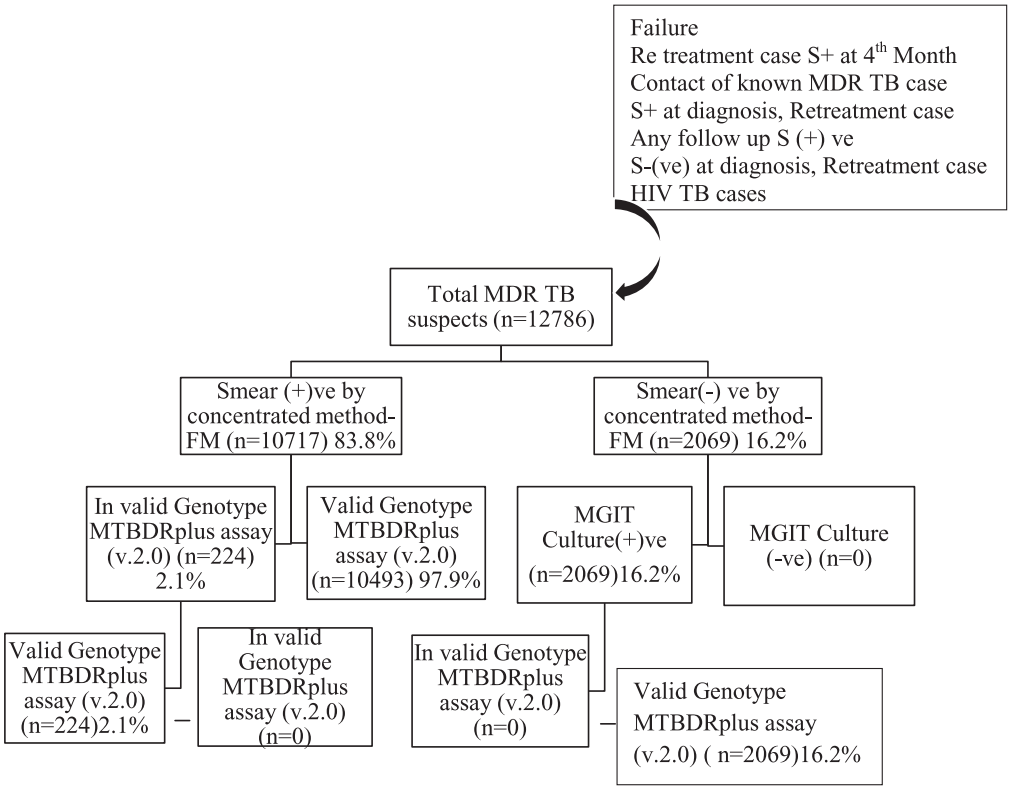


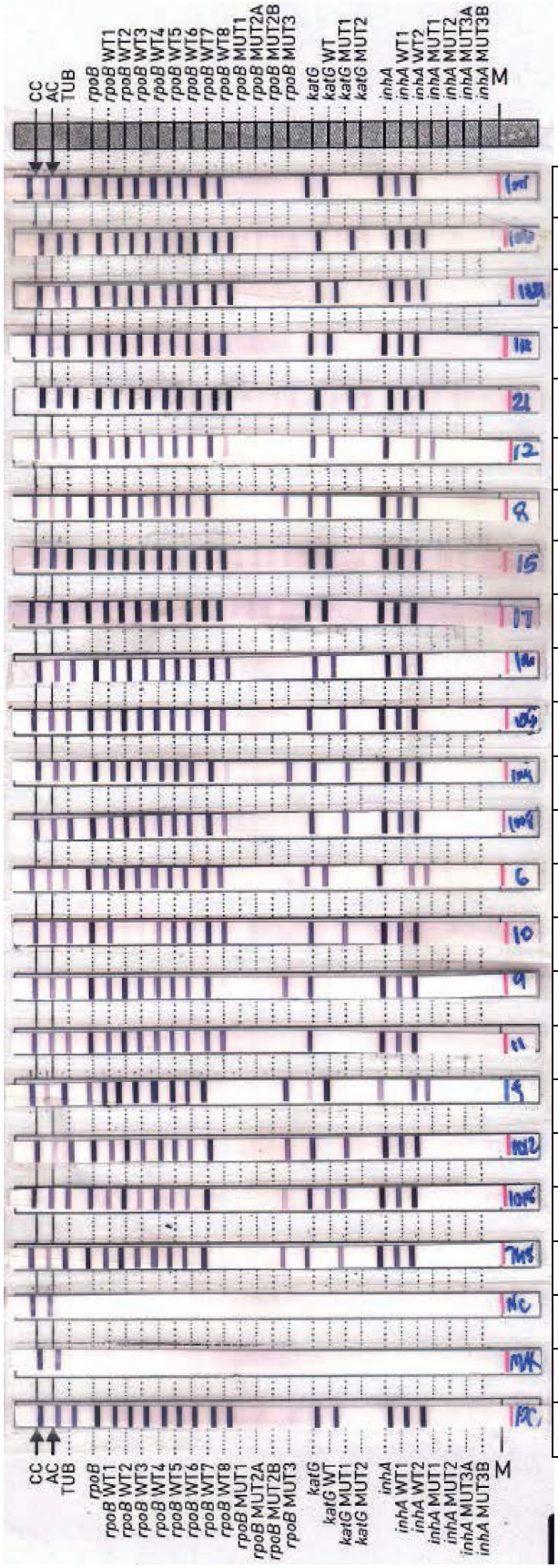
Fig. 1. Specimen flow schematic.

Table 2
MDR TB diagnosed on gender and age wise.

Criteria	Sex	Total MDR TB diagnosed on age and criteria basis								Total MDR TB		Grand total
		≤15 years		>15 ≤ to 45 years		>45 to ≤60 years		>60 years				
		RH	R	RH	R	RH	R	RH	R	RH	R	
Failure	Male	0	0	25	17	17	5	3	1	45	23	68
	Female	0	0	2	3	0	2	0	0	2	5	7
Re treatment case S+ at 4th month	Male	0	0	10	10	9	6	2	1	21	17	38
	Female	1	0	7	2	0	2	1	1	9	5	14
Contact of known MDR TB case	Male	0	0	7	1	1	1	0	0	8	2	10
	Female	0	0	4	0	1	0	0	0	5	0	5
S+ at diagnosis, re treatment case	Male	0	2	105	83	78	45	29	14	212	144	360
	Female	0	0	39	12	14	8	3	1	56	21	77
Any follow up smear positive	Male	0	0	73	19	34	18	14	6	121	43	164
	Female	0	0	20	1	9	3	0	0	29	4	33
S - at diagnosis, re treatment case	Male	0	0	15	4	9	3	2	1	26	8	34
	Female	0	0	4	3	1	1	0	0	5	4	9
HIV TB case	Male	0	0	11	10	3	4	1	0	15	14	29
	Female	0	1	4	2	0	0	0	0	4	3	7
Total	Male	0	2	246	144	151	82	51	23	448	251	699
	Female	1	1	80	23	25	16	4	2	110	42	152
Grand total		1	3	326	167	176	98	55	25	558	293	851

conventional BACTEC MGIT 960 culture procedures. Overall, 83.8% smear positive specimen's results effectively including the results of repeated testing in 224 (2.1% invalid) cases as shown in Fig. 1. The results of Genotype MTBDRplus V.2.0 assay is interpreted as shown in the Fig. 2.

Among 851 diagnosed MDR tuberculosis, 90% cases were diagnosed from age group of >15 to ≤45 years and >45 to ≤60 years respectively (Fig. 3) and rifampicin mono resistant were diagnosed equal proportion to multi drug resistant from these age group only (Fig. 4). The diagnosed MDR TB cases in pediatric age group were 0.47% and 9.4% MDR TB cases were diagnosed in the age group of >60 years. The 50.5% mutations were observed in the region of S531L in MDR TB patients and 55.6% in rifampicin monoresistant cases (Fig. 5). In total isoniazid monoresistant, 68.0% mutations were detected in *katG* gene, which is more prevalent in comparison to *inhA* gene 32.0% (Fig. 6). In isoniazid monoresistant strains, the particular type of mutations were observed uniformly, which highly specified the efficacy of the detection of mutations in *katG* and *inhA* genes. This difference in prevalence of mutations in MDR strains compared with INH monoresistant strains was significant for *katG* but not in *inhA*. Surprisingly, only two strains had mutations in both the *katG* and *inhA* genes.



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+	-	+	+	+	+	-		R		R
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Fig. 2. The interpretation of LPA results with various resistant/sensitive pattern.

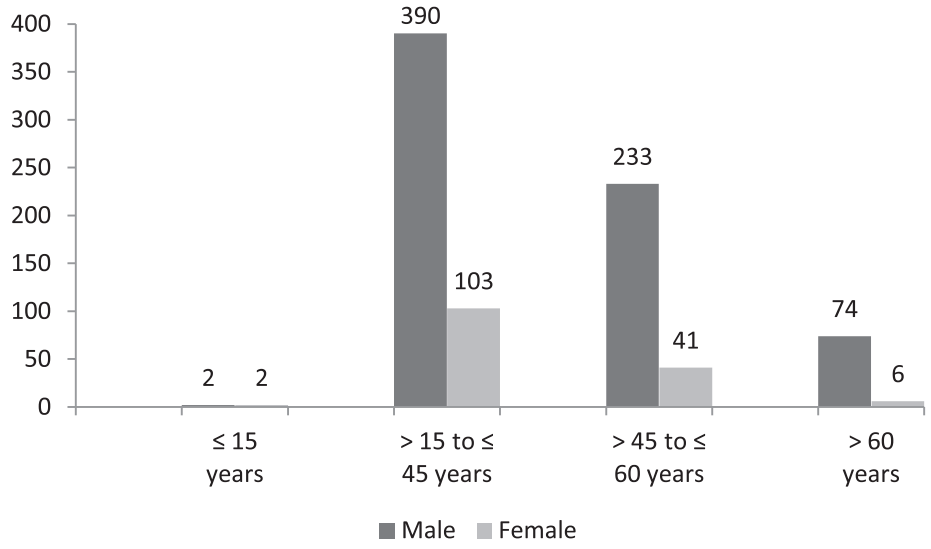

Fig. 3. Total no of multi drug resistant on age and sex wise.

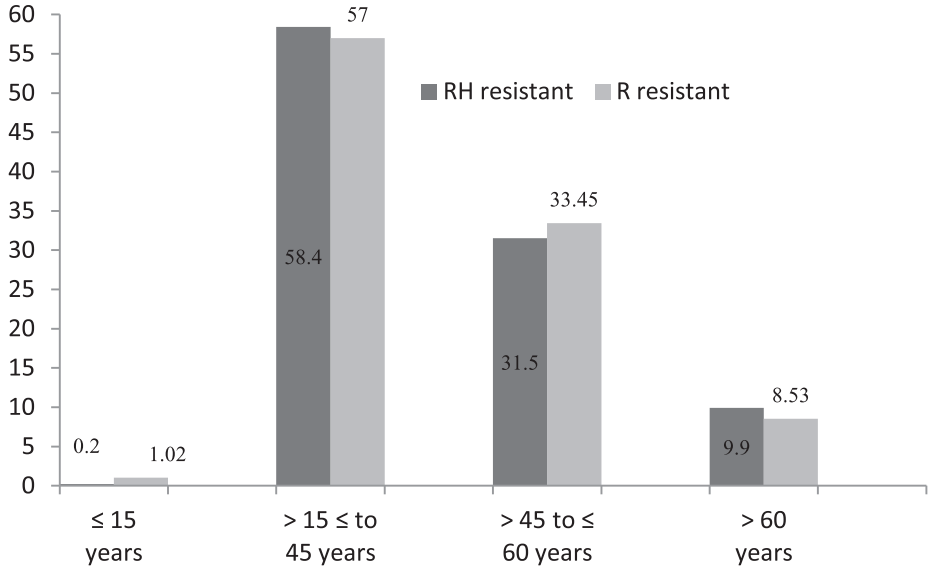

Fig. 4. Percentage of multi drug and Rif mono resistant on age wise.

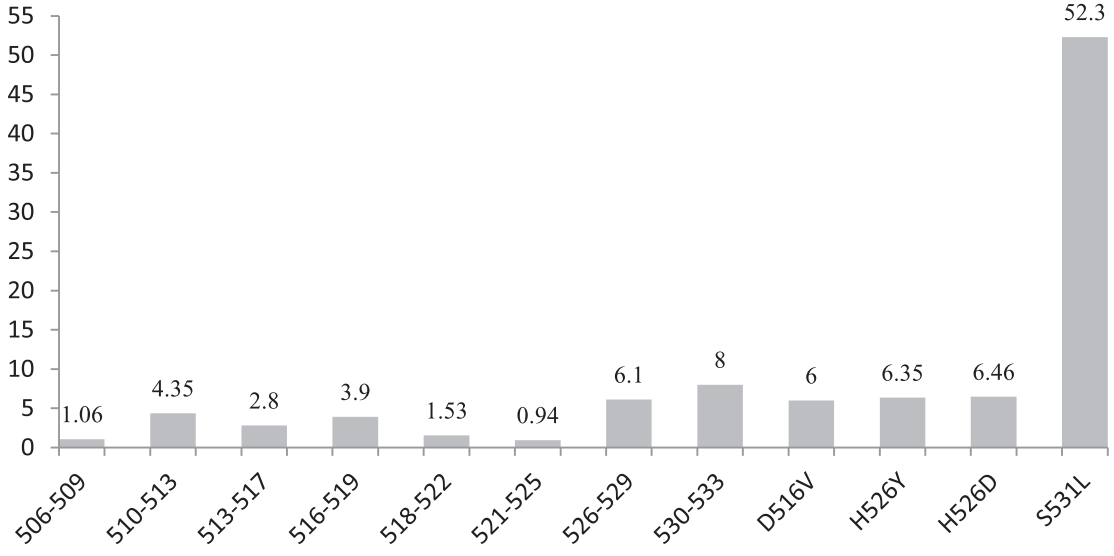

Fig. 5. Frequency of mutations on codons of hyper variable region of *rpoB* gene.

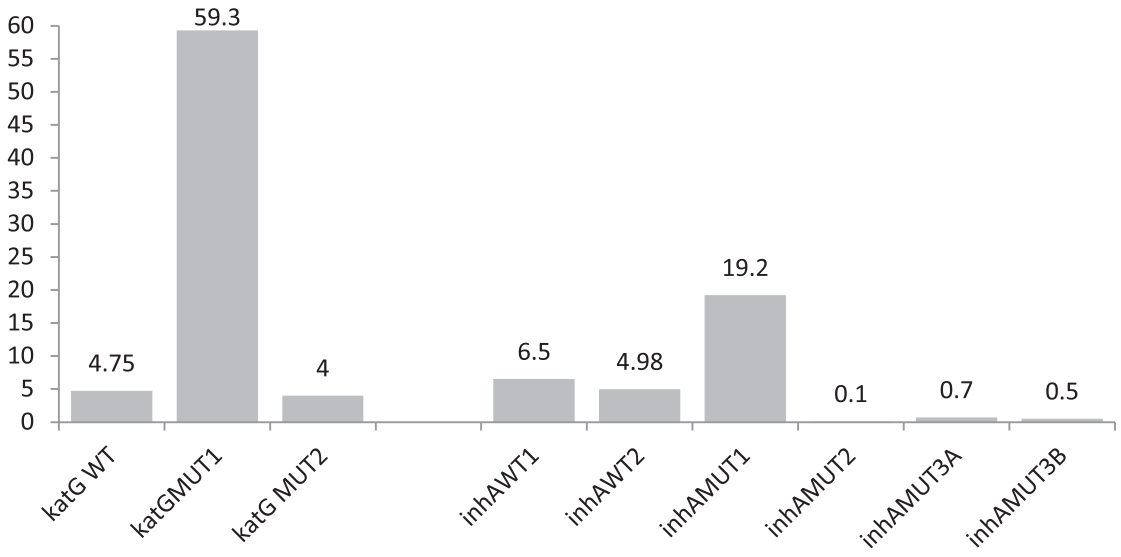

Fig. 6. Frequency of mutations on codons of *katG* and *InhA* genes.

Discussion

In this study, the performance of the GenoType MTBDRplus V.2.0 assay was assessed that offers the simultaneous identification of *M. tuberculosis* and its resistance to rifampicin (RIF) and isoniazid (INH) by detecting the most common mutations in the *rpoB* and *katG* genes. As reported widely elsewhere, rifampicin resistance was highly associated with mutations in the 81 base pair region of the *rpoB* gene [35,36]. DNA sequencing studies have shown that greater than 95% of the RIF-resistant strains have mutations within an 81 base pair hot-spot region (codons 507–533) of the *rpo*B gene [9]. Though more than 50 mutations within this region have been characterized by automated DNA sequencing, the majority involve point mutations at codons 516, 526, or 531 [1]. Priyanka et al. [27] found a higher (89.65%) proportion of RMP resistance due to S531L mutations. Barnard et al. [5] also found higher proportion (70.5%) RMP resistance due to S531L in their study. Similar to both above mentioned studies, Ravindran et al. (20,012) also found codon 531 of the rpoB was the most frequently encountered (84.6%). Raj et al. [28] found 72% RMP resistance due to S531L in their study. But we found a 52.3% proportion of RMP resistance due to S531L mutations. The reasons for lesser proportion of RMP resistant due to S531L mutations are sample volume and selection of sample criteria. A merely equal proportion of rifampicin resistance (52.3%) due to S531L mutations as what has been reported in other geographical locations (between 36.1 and 56.7%) [17,22]. Viveiros et al. [38], Marinus et al. [20], Florence et al. [14] reported the "false" resistant strains in their study as absence of RIF WT2 probe, interestingly; the same sort of mutation was found in this study made justification for the possibility of true resistance as of 4.3% in MDR cases and two (4.4%) in RIF-mono resistant strains. These results indicated that the GenoType MTBDRplus V.2.0 assay is also capable of revealing the presence of rare mutations.

The various types of uncommon drug resistant patterns identified are; thirteen MDR strains had absence of WT8 and a H526Y mutation, five MDR strain had both H526Y and S531L mutations, four MDR strain had H526Y and H526D, two MDR strain had a H526D and S531L mutation. Other mutations in the 530–533 regions were common, as detected by the lack of binding with the WT8 probe in the presence of S531L mutation. A significantly higher proportion of RIF-monoresistant strains (10.6%) had a lack of binding with the WT8 probe and in combination with presence of MUT3 (S531L) probe (55.6%), compared with MDR strains (50.5%). There was no significant difference in the presence of other bands between MDR and RIF monoresistant strains. In previous study, Akos Somoskovi et al. [2] reported a rare mutation of WT2 (Q513L mutation), as a "false" RIF-resistant. The same mutation of WT2 probe was missing observed in thirty seven strains in this study also. But it was combined with absence of *katG* WT probes in thirteen strains and presence of MUT1 (S315T) probe in twenty four strains, thus made these strains ensemble are indicative of true resistant.

The prevalence of mutations in the *katG* and *inhA* genes seems to vary widely in different geographic locations [2]. It was observed that the prevalence of mutations in *katG* gene plays major role in determining of MDR-TB, when compare to *inhA* gene. However, in concordant with other studies, it was observed that the most common mutations at codons *katG* WT: 315, MUT1: S315T and *inhA* WT: −15/−16, MUT1: C15T. It was established that a higher proportion of INH resistance due to MUT1: S315T probe [19]. Studies from other countries have confirmed this variability in the contribution of different mutations to INH resistance [4]. In rare case six INH monoresistant had a MUT3A (T8C) mutation and it has not been published elsewhere. A high prevalence of *katG* mutations has been reported to account for a high proportion of MDR and INH-resistance in high-TB prevalent countries and for a much lower proportion in lower-TB prevalent settings, presumably due to on-going transmission of these strains in high-burden settings [31,23]. In INH-resistant cases region of wild types in *katG* and *inhA* genes showed repeated mutations.

Conclusion

In conclusion, the rate of occurrences of mutations were found widely in the Rifampicin Resistant Determination Region (81 bp) of *rpoB* gene and the hypervariable region 530–533 codons of *rpoB* gene is alarming in the specification. The most frequent mutations involved in resistance to Rifampicin and Isoniazid drugs was observed in this study and this higher frequency of mutation in particular codons of *rpoB* (S531L) and *katG* (S315T) gene help to design new simple less expensive molecular assay to use at peripheral laboratories in developing countries.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Informed consent

Not applicable.

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