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Molecular Genetic Determination of MDR – TB Isolates from West Bengal, India

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Abstract

Tuberculosis (TB) is one of the leading causes of death from an infectious disease worldwide. India remains to be the country carrying highest burden of the disease. One of the potent problems of present days, in this field, is the emergence of Multi-Drug-Resistant Tuberculosis (MDR-TB). MDR is defined as resistance to isoniazid (INH) and rifampin (RIF). The spread of MDR-TB is one of the biggest challenges to global public health system. Thus, detection of MDR-TB strains is critically important for containment of global TB epidemics. In this study, we are determining the MDR incidence rate in the West Bengal state of India, which is one of the most TB prone areas of the world. The RIF-resistant mutations of *Mycobacterium tuberculosis* (MTB) were shown to map in the *rpoB* locus and INH-resistant mutations of MTB were shown to map in *inhA* and *katG* loci. Therefore, in the present study, we have detected MDR-TB strains by mapping *rpoB*, *katG* & *inhA* mutations. Our Line Probe Assay (LPA) based results from a vast pool of MTB organisms (carrying 3653 bonafide sputum positive patient isolates) indicated the presence of 14.37% MDR isolates which matches in general with the previously reported results from various parts of the world (approx. 12-16%). Therefore, we conclude that our MDR rate is generally comparable to that of the other investigations performed in the rest of the world. We also found that the male patients are more likely to contract the MDR strains than the female patients (1.97:1.0). This data also conforms to the global statistics.

Keywords: Tuberculosis; MDR-TB; PCR; LPA; GenoType MTBDRplus Assay; RpoB; InhA; KatG

Introduction

Tuberculosis (TB) is a leading Public health problem globally [1]. A total of 2-3 billion people are infected with tuberculosis (TB); of this huge infected population 5-10% will develop the disease tuberculosis. According to latest WHO statistics 10.4 million new TB cases have been reported in the year 2016. There were also 1.8 million deaths causes by TB. Therefore it is a leading cause of infectious death. Currently, out of 1.8 million TB deaths, nearly 1.4 million were HIV negative & 0.4 million patients were HIV positive. Hence, TB is the primary opportunistic infection for HIV positive patients. All of the aforementioned facts indicate that TB is one of the greatest challenges to the current global public health system. Roughly one forth, of the globally reported TB cases occurs in India annually [2]. Thus, performing the studies of TB in this country is immensely important. Multidrug-Resistant Tuberculosis (MDR-TB) is defined as resistant to Isoniazid (INH) and Rifampin (RIF), with or without resistance to other First-Line-Drugs (FLD) [3]. MDR-TB must be treated with so-called "second-line" drugs [4] which are less effective, more expensive, and associated with more serious side effects than first-line treatments. Due to this reason, global tuberculosis control programme are severely compromised by increased frequency of generation of MDR stains. That is why, it is very important to detect MDR-TB stains quickly. As the diagnosis by the classical culturing methods require anywhere from 6 to 16 weeks as well as sophisticated laboratory equipment, thus now-a-days TB drug resistance is detected by Nucleic Acid Amplification Tests (NAATs) [5]. Line Probe Assay (LPA) [6] is one of the currently used cutting-edge techniques based on NAAT, which is used for the detection of INH-resistance, RIF-resistance as well as MDR-genotypes on a global scale. In this report, LPA technique has mainly been used to profile the MDR-TB strains in West Bengal state of INDIA. It has been shown that, INH-resistance results from both *inhA* and *katG* mutations [7,8]. *inhA* is the target for the drug INH. *inhA* promoter up mutations result in the INH resistance, by producing the encoded InhA proteins in high concentration. In rare cases, structural mutation in *inhA* also gives rise to INH-resistance [7,9]. *KatG* is required for activation of drug INH [8,10]. Hence, several structural mutations in *katG* lead to INH-resistance. The target for RIF is RNA polymerase which is coded by *rpoB*. Several *rpoB* mutations account for all of the RIF-resistance in MTB. Therefore, for screening of MDR-TB isolates, *inhA*, *katG* and *rpoB* probes are all used together in a NAAT based protocol. In the current study, we report characterization of the MDR-TB isolates mainly by LPA techniques from a vast pool of TB specimens that are collected from the West Bengal province of INDIA which is a highly TB-prone area in the world. In this report, we compared our collected data with that of pre-existing global data, to elucidate certain important trends regarding MDR-TB statistics.

Materials and Methods

Specimen collection, processing and data analysis

Firstly, the sample collection, record keeping, Line Probe Assay (LPA) and data reporting were done following Revised National Tuberculosis Control Program (RNTCP) protocols [3,11]; all work has been carried out in the Intermediate Reference Laboratory (IRL) in Kolkata under the RNTCP umbrella following ethical principles and human subject approvals as per RNTCP guidelines.

The retrospective record based study includes information on cultures and Drug Susceptibility Testing (DST) outputs of the sputum samples obtained from all smear positive patients. These samples were received at the IRL Kolkata from different parts of West Bengal state between January 2015 and October 2015. The clinical data, obtained from all the TB-patients, were recorded in accordance with the standard RNTCP protocols [3,11].

A summary of the clinical isolates collected from the patients are described in Table 1 and their age-wise distribution is shown in Table 2 And, the drug-resistance profiles of these isolates have been given in Table 3. Two sputum samples from each patient were collected and processed following a modification from previously published protocols [3,12,13].

Total no. of all TB patients		Total no. of male TB patients		Total no. of female TB patients		No. of MDR-TB patients		No. of mono INH-R TB Patients		No. of mono RIF-R TB Patients		No. of pan sensitive TB Patients		No. of HIV-positive TB Patients		No. of HIV-positive MDR - TB Patients	
3653 (100%)		2101 (57.51%)		1552 (42.49%)		525 of 3653 (14.37%)		365 of 3653 (9.99%)		72 of 3653 (1.97%)		2691 of 3653 (73.66%)		216 of 3653 (5.91%)		09 of 525 (1.71%)	
NSP	RT	NSP	RT	NSP	RT	NSP	RT	NSP	RT	NSP	RT	NSP	RT	NSP	RT	NSP	RT
596 (100%)	3057 (100%)	325 of 596 (54.53 %) [male NSPs / all NSPs]	1776 of 3057 (58.09 %) [male RTs/ all RTs]	271 of 596 (45.47 %) [female NSPs/ all NSPs]	1281 of 3057 (41.9%) [female RTs/ all RTs]	14 of 596 (2.35 %)	511 of 3057 (16.72 %)	52 of 596 (0.84 %)	313 of 3057 (10.24 %)	07 of 596 (1.17 %)	65 of 3057 (2.13 %)	523 of 596 (87.75 %)	2168 of 3057 (70.92 %)	121 of 596 (20.30 %)	95 of 3057 (3.10 %)	5 of 596 (0.84 %)	4 of 3057 (0.13 %)
		325 of 2101 (15.47 %) [male NSPs / all male patients]	1776 of 2101 (84.54 %) [male RTs/ all male patients]	271 of 1552 (17.46 %) [female NSPs/ all-female patients]	1281 of 1552 (82.54 %) [female RTs/ all-female patients]	M	F	M	F								
						8	6	334	177								

Table 1: Patient Characteristics. MDR frequency is calculated in terms of percentage based on a total pool of 3653 TB isolates. NSP, new sputum positive; RT, retreatment cases. M, male; F, female. INH-R, isoniazid-resistant; RIF-R, rifampin-resistant

Age Group	No. of Male TB Patients	No. of Female TB Patients	No. of male patients with MDR-TB	No. of Female patients with MDR-TB	Total % of MDR-TB
0-10	0	1	00 [of 0] (%)	0 [of 1] 0%	0
11-20	221	201	48 [of 221] (21.72%)	21 [of 201] (10.45%)	13.82%
21-30	442	455	72 [of 442] (16.29%)	52 [of 455] (11.43%)	13.82%
31-40	420	385	59 [of 420] (14.05%)	31 [of 385] (8.05%)	11.18%
41-50	502	232	69 [of 502] (13.75%)	29 [of 232] (12.5%)	13.35%
51-60	352	120	56 [of 352] (15.9%)	34 [of 120] (28.33%)	19.09%
61-70	135	95	28 [of 135] (20.74%)	08 [of 95] (8.42%)	15.65%
71-80	24	49	08 [of 24] (33.33%)	02 (of 49) (4.08%)	13.69%
81-90	5	14	02 [of 05] (40%)	06 [of 14] (42.86%)	42.10%

Table 2: Age-based distribution of the MDR-TB incidence rate. Age groups were divided decade-wise. From respective age groups, MDR frequencies were calculated in terms of percentage of total MDR male or female patients and their respective age groups.

Gene	Band	Codons Analyzed/ Mutations	Codon Substitutions	MDR-TB Strains (n=525)	INH Monoresistant Strains (n=365)	RIF Monoresistant Strains (n=72)	Pan-sensitive Strains (n=2691)	P-value (<0.01)
rpoB								
	WT1	505-509	-	525 (100%)	365 (100%)	72 (100%)	2691 (100%)	-
	WT2	510-513	-	520 (99.04%)	365 (100%)	68 (94.44%)	2691 (100%)	0.003515
	WT3	513-517	-	449 (86.51%)	365 (100%)	64 (72.22%)	2691 (100%)	0.446657
	WT4	516-519	-	449 (85.52%)	365 (100%)	64 (51.38%)	2691 (100%)	0.446657
	WT5	518-522	-	525 (100%)	365 (100%)	72 (100%)	2691 (100%)	-
	WT6	521-525	-	525 (100%)	365 (100%)	72 (100%)	2691 (100%)	-
	WT7	526-529	-	459 (87.43%)	365 (100%)	61 (77.77%)	2691 (100%)	0.520354
	WT8	530-533	-	102 (19.43%)	365 (100%)	23 (38.88%)	2691 (100%)	0.014365
	MUT1	D516V	GAC→GTC	76 (14.48%)	00 (%)	08 (11.11%)	00 (%)	0.965223
	MUT2A	H526 Y	CAC→TAC	23 (4.38%)	00 (%)	09 (12.50%)	00 (%)	0.003927
	MUT2B	H526 D	CAC→GAC	38 (7.24%)	00 (%)	02 (2.77%)	00 (%)	0.154765
	MUT3	S531 L	TAG→TTG	369 (70.29%)	00 (%)	41 (56.94%)	00 (%)	0.022138
katG								
	WT	315	-	138 (26.29%)	109 (29.86%)	72 (100%)	2691 (100%)	0.120799
	MUT1	S315 T1	AGC→ACC	403 (76.76%)	268 (70.14%)	00 (%)	00 (%)	0.255204
	MUT2	S315T2	AGC→ACA	04 (0.76%)	02 (0.54%)	00 (%)	00 (%)	0.690188
inhA		Analyzed N.A. Position	Type of Mutation					
	WT1	-15/-16	-	400 (79.19%)	273 (66.30%)	72 (100%)	2691 (100%)	0.632437
	WT2	-8	-	502 (95.62%)	348 (89.86%)	72 (100%)	2691 (100%)	0.843515
	MUT1	C(-15)T	Promoter Up-Mutation	122 (23.24%)	91 (17.33%)	00 (%)	00 (%)	0.560567
	MUT2	A(-16)G	Promoter Up-Mutation	03 (0.57%)	01 (0.27%)	00 (%)	00 (%)	0.509318
	MUT3A	T(-8)C	Promoter Up-Mutation	21 (4.00%)	16 (4.38%)	00 (%)	00 (%)	0.780245
	MUT3B	T(-8)A	Promoter Up-Mutation	02 (0.38%)	01 (0.27%)	00 (%)	00 (%)	0.778404

Table 3: Line probe assay based band pattern analysis of drug-resistant *Mycobacterium tuberculosis* strains. N.A., nucleic acid; n, number of *M. tuberculosis* strains analyzed.

Mycobacterial DNA extractions in BSL-3 laboratory as well as the Geno-Type MTBDRplus LPA manipulations [6] were performed in accordance with the manufacturer's (Hain Life Science, Nehren, Germany) instructions [6] and the RNTCP protocol [3]. LPA Tests were performed, via a 3-step procedure, which includes DNA extraction, multiplex PCR amplification and reverse hybridization. These 3 steps were executed in 3 separated work areas. The protocol of the LPA analysis followed in our laboratory is described below:

Line probe assay (LPA)

DNA extraction

Aliquots of 500 µl each of decontaminated sputum samples were put into the 1.5 ml centrifuge tubes. After 15 min centrifugation at 11,700 g, the pellets were resuspended into 100 µl lysis buffer and heated at 100 °C for 10 min. Next, the lysates were spun down. Then, to each tube of lysate, 100 µl of the neutralization buffer was added and mixed well. Each neutralized lysate was centrifuged for 5 minutes at 11,700 g.

100 µl of the supernatant was collected into a microfuge tube and 5 µl of this liquid was used for PCR amplification (3,6,12,13,14,15).

PCR amplification

The amplification mix A was constituted of 10 X buffer (5 µl PCR buffer containing 15 mM $MgCl_2$), oligonucleotides (2 µl mixture of ddATP, ddTTP, ddGTP and ddCTP containing 25 mM $MgCl_2$), 0.2 µl Taq polymerase and 2.8 ml dH_2O. Amplification mix B contained $MgCl_2$, the biotinylated primers (for details, see below) and dye. For setting up one PCR reaction, 10 ml of amplification mix A was gently mixed with 35 ml amplification mix B by inverting the tube several times to constitute the master mixture. 5 ml of each DNA template obtained from the DNA extraction protocol mentioned above was added to each PCR tube containing 45 ml of master mixture and mixed gently. Thereafter, the PCR tubes were placed into the thermal cycler from Hain Lifesciences with the following cycling parameters: 1) 15 min at 95 °C – 1 cycle; 2) 30 sec at 95 °C followed by 2 min at 65 °C – 20 cycles; 3) 25 sec at 95 °C, 40 sec at 50 °C and 40 sec at 70 °C – 30 cycles; 4) 8 min at 70 °C – 1 cycle. Then, amplification products were stored at -20 °C. The PCR amplification reaction was performed using the forward *rpoB* primer 5'-CGACCACTTCGGCAACCG-3' and reverse *rpoB* primer 3'-TCGATCGGGCACATCCGG-5'; the forward *katG* primer 5'-TCGGCGGTCACACTTTCGGTAAGA-3' and the reverse *katG* primer 3'-GCGACGCGTGATCCGCTCATAG-5'; the forward *inhA* primer 5'-CGAGCGTAACCCCAGTGCGAAAGT-3' and the reverse *inhA* primer 3'-CCCCGGTGAGGTTGGCGTTGAT-5' (12).

Hybridization

The Genotype MTBDR assay was performed following the manufacturer (Hain Life Sciences, Germany) supplied protocols using the reagents provided in the kits. The process is clearly described in the instruction manual [6] as well as in prior publications [12,13,15]. The hybridization step employs many probes to verify several aspects of the molecular assay [6,13,15]. Besides having the *rpoB* probes (Figure 1A) for monitoring Rif-R status of an isolate, *inhA* and *katG* probes are included to monitor the INH-R status; appearance of their respective band hybridization patterns (Figure 1B and Figure 2) indicates sensitivity/resistance to RIF and INH, respectively. Conjugate control (CC), amplification control (AC) and tuberculosis bacilli (TUB) are control probes. For instance, the TUB probe indicates the presence of a sample isolated from the *M. tuberculosis* complex. *rpoB*, *katG* and *inhA* probes are the locus amplification control probes for indicating presence of respective genes.

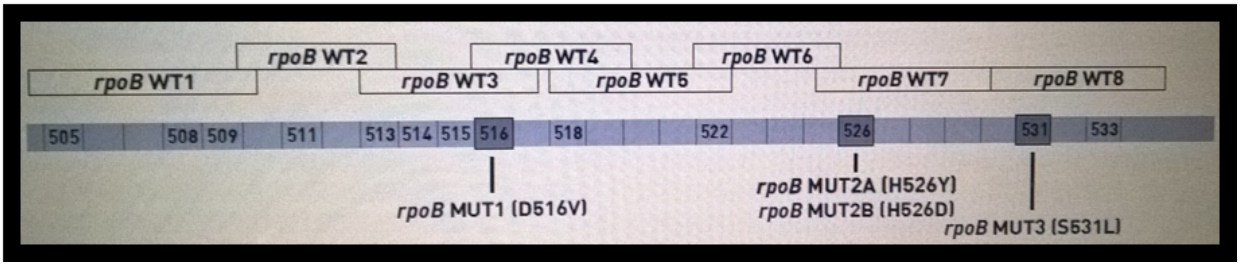

Figure 1A: Map of *rpoB* probes. *rpoB* WT1-8 are the *rpoB* wild type probes, *rpoB* MUT1-3 are the probes for detection of *rpoB* mutants, conferring RIF-resistance. The numbers specify the positions of the amino acids/codons for mutations. The codons for which the mutation probes were designed are indicated by labelling. (This figure has been adopted from the manufacturer-supplied manual for GenoType MTBDRplus 2.0 kit [11]).

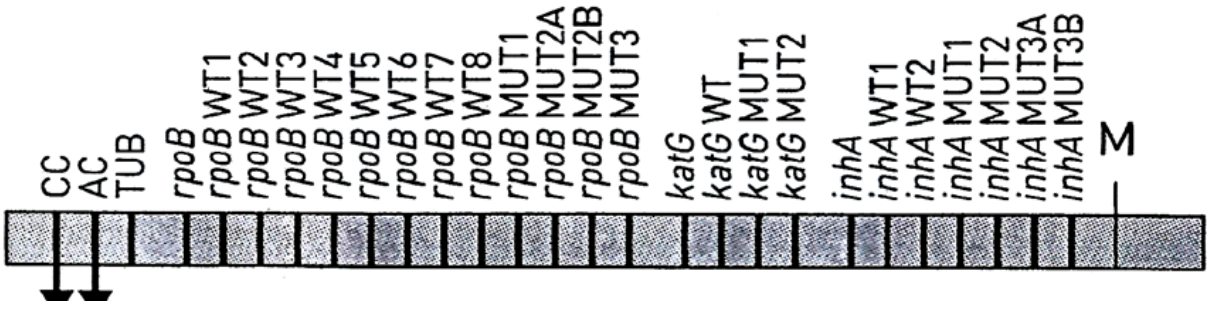

Figure 1B: Band patterns of line probe assay (GenoType MTBDR-plus) strip. CC, conjugate control; AC, amplification control; TUB, tuberculosis bacilli complex; *rpoB*, locus control for *rpoB* gene; *rpoB* (WT1-WT8), *rpoB* wild type probes 1-8; *rpoB* (MUT1-MUT3), *rpoB* mutation probes 1-3; *katG*, locus control for *katG* gene; *katG* WT, *katG* wild type probe; *katG* (MUT1 and MUT2), *katG* mutation probes 1 and 2; *inhA*, locus control for *inhA* gene; *inhA* (WT1 and WT2): *inhA* wild type probes 1 and 2; *inhA* (MUT1-MUT3B): *inhA* mutation probes 1-3B; M, colored marker.

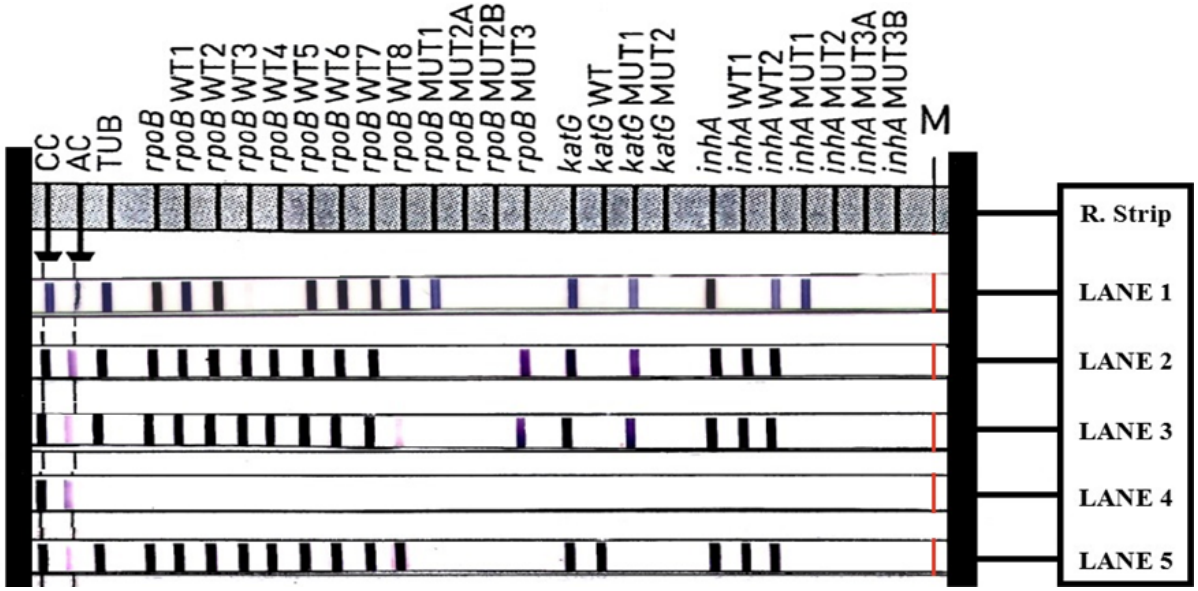

Figure 2: Representative patterns of line probe assay using GenoType MTBDRplus VER 2.0 kit. Lane 1, MDR-TB isolate #1 [with *rpoB* MUT1 i.e. D516V mutation {because WT3 & 4 bands are absent with presence of MUT3 band}, *katG* MUT1 i.e. S315T2 mutation {because WT band is absent with presence of MUT1 band} and *inhA* MUT1 i.e., C(-15)T mutation {because WT1 band is absent with presence of MUT1 band}]; Lane 2, MDR-TB isolate #2 [with *rpoB* MUT3 i.e. S531L mutation {because WT8 is absent with presence of MUT3 band} and *katG* MUT1 i.e. S315T1 mutation {because WT band is absent with presence of MUT1 band}]; Lane 3, MDR-TB isolate #3 [with *rpoB* MUT3 i.e. S531L mutation and *katG* MUT1 i.e. S315T1 mutation) because of band patterns similar to that of Lane 2; Lane 4, DNA negative control; Lane 5, H37Rv as DNA positive control (sensitive to rifampicin and isoniazid – hence, with all WT bands and no mutant bands).

Other than the aforementioned six control probes, the hybridization signals generated using the twenty-one experimental probes for mutation detection (Figure 1B and Figure 2) are used to indicate the presence/absence of mutations in the genes *rpoB*, *katG* and *inhA*. For example, there are 12 probes which were used for *rpoB* mutational detection; they are numbered WT1-8 as well as MUT1-3 with their maps shown in Figure 1A. The maps of *katG* and *inhA* probes are not shown here but their designing principle is similar. All *katG* probes are for scoring mutants mapping at codon 315. *katG* WT band (Figure 2) lights up if the sequence is wild-type whereas *katG* MUT1 or *katG* MUT2 signals are produced when the H315Y or the H315D mutations (Table 3) are present, respectively. With an analogous strategy, *inhA* WT1, *inhA* MUT1 and *inhA* MUT2 probes are designed for scoring the promoter-up mutations mapping at positions -15/16 of *inhA*. Wild-type sequence produces signals with *inhA* WT1 probe whereas *inhA* MUT1 generates signal for C(-15)T mutant and *inhA* MUT2 produces signal for A(-16)G mutant (Table 3). Again, *inhA* MUT3A and *inhA* MUT3B probes produce signals for T(-8)C mutant and T(-8)A mutant, respectively, whereas *inhA* WT1 generates signal with the wild-type sequence (Table 3). The hybridization strips used in the reverse hybridization step consist of twenty-seven potential reaction zones -6 for control probes and 21 for mutational detection (Figure 1B). Only a subset of these 27 bands can be found in each sample lane/strip (Figure 2); according to the observed band patterns, the clinical samples were scored (Table 3) for INH resistance/sensitivity and RIF resistance/sensitivity [6,13,15].

In this assay, if the TB isolate bears a wild-type (RIF-sensitive) *rpoB* locus, the expected band pattern generated by the hybridization assay should be WT1-8 (all the 8 wild-type bands) with no corresponding signals for mutational bands (no MUT1-3 signals). In case of isolates containing mutant *rpoB* genes (RIF-resistant), MUT1/MUT2A-MUT2B/MUT3 should appear instead of WT3&4/WT7/WT8 (Figure 2). Similarly, the INH resistance can be determined by analyzing the hybridization band patterns corresponding to *katG* and *inhA* loci. For instance, *katG* mutations would be detected by presence/absence of the WT hybridization band vis-à-vis the band for MUT1 and MUT2 (Figure 1A). Mutations in *inhA* locus would be detected by presence/absence of WT1 and WT2 bands as opposed to MUT1&MUT2/MUT3A&MUT3B (Table 3). During the study period, both Internal Quality Control (IQC) and External Quality Control (EQC) were included. Control strain H37RV (ATCC27294) was used for IQC. EQC of the laboratory was done by retesting and panel culture testing by the National Tuberculosis Institute (NTI) Bangalore, which is a World Health Organization (WHO) collaborating National Reference Laboratory (NRL). During the study period, IRL Kolkata laboratory recorded >90% proficiency in INH and RIF resistance evaluation using NRL controls which were within the acceptable limit of the quality assurance assessment [2]. All the Line Probe Assay related statistical analysis was performed using standard methodologies [16].

Result

Frequency of drug-resistant tuberculosis

Among 3653 isolates (Table 1) examined in this study (all of which were obtained from pulmonary TB patients), 596 patients were characterized as New Sputum Positive (NSP). NSP patients were

described as those who never had any treatment for TB or, had a course of anti-TB drugs for less than 4 weeks. These patients may have positive or, negative results from bacteriological tests. The rest of the isolates (3057) were from Re-treatment (RT) patients. RT cases are defined as those, who have received a course of Anti-TB drugs for at least 1 month or, greater. These patients can also have positive or, negative bacteriology similar to NSP [17].

Notably, 525 of all (3653) isolates (14.37%) were characterized by LPA methodology as MDR-TB (i.e., resistant to both INH and RIF). Of all NSP cases (596 isolates), 14 (2.35%) were characterized as MDR-TB. Of all RT cases (3057 isolates), 511 (16.72%) were found to be MDR-TB.

Among male pool (2101 isolates, which is 57.51% of all patients included in the study), there were 325 NSP isolates (15.47% of all male isolates). The rest of the male pool isolates (i.e., 1776 isolates [84.54% of all male isolates]) are from RT cases. Based on the aforementioned counts, 16.28% (342 of 2101) of the male pool are characterized as MDR-TB isolates. Among male NSP cases (325 isolates in total), MDR-TB isolates numbered in 8. Therefore, 2.46% (8 of 325) of NSP-male cases are MDR-TB. Among male RT cases (numbering 1776), the number of MDR-TB isolates is 334. Therefore, 18.80% (334 of 1776) of RT-male cases are MDR-TB.

Among female pool (1552 isolates, which is 42.49% of all patients included in the study), there were 271 NSP isolates (17.46% of all female isolates). The rest of the female pool isolates (i.e., 1281 isolates [82.54% of all female isolates]) are from RT cases. Based on these counts, 11.79% (183 of 1552) of the female pool are characterized as MDR-TB. Among female NSP cases (271 isolates in total), MDR-TB isolates numbered in 6. Therefore, 2.21% (6 of 271) of NSP-female cases are MDR-TB. Among female RT cases (1281 isolates in total), MDR-TB isolates number in 177. Therefore, 13.81% (177 of 1281) of RT-female cases are MDR-TB.

Hence, comparing the data presented in the two paragraphs presented above, the frequency of MDR causation in male patients is much higher than that of the female patients in every sense (Table 1). For instance, the MDR-frequency of all male cases is 16.28% (342 of 2101) whereas that of all female cases is 11.79% (183 of 1552). If we consider only the NSP cases, the MDR-frequency of the male pool is 2.46% (8 of 325) whereas that of the female pool is 2.21% (6 of 271). If we consider the RT cases, the MDR-frequency of the male pool is 18.80% (334 of 1776) whereas that of the female pool is 13.81% (177 of 1281). Comparing the data from every category, the male patients seem to have a higher MDR conversion rate than the female patients. In sum, we observe a sex-wise difference in MDR conversion rate with male TB-patients being more prone to carry MDR-TB than female.

We also have tried to find out if there exists any age-wise difference in MDR-conversion rate by grouping TB-patients in age-brackets of 10 years (Table 2); the patients were distributed in 9 categories ranging from 0-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80 and 81-90. From our age-wise distribution results, extremely high MDR-conversion is observed in very old-aged patients (in the 81-90 years age-group); however, it has to be noted that the number of patients in this age-group is too small to draw any meaningful conclusion. Otherwise, MDR-conversion does not show any definitive pattern over different age categories. However, in age group 51-60, the MDR-conversion rate is much higher in females than that of males. This observation is against the general trend of males having higher MDR-rate than that of females.

365 (9.99%) isolates of 3653 were characterized as mono INH-resistant (INH-R) TB patients. Among these INH-R mono-resistant isolates, 0.84% (52 out of 596) patients were NSP and 10.24 % (313 out of 3057) were RT. All of these were found to be RIF-sensitive by our LPA characterization. 72 (1.97%) isolates of 3653 were characterized as mono RIF-resistant (RIF-R) TB patients. Among these RIF-R isolates, 1.17% (7 out of 596) were NSP and 65 out of 3057 (2.13%) were RT. All of these were found to be INH-sensitive by LPA characterization.

Pan-sensitive isolates were sensitive to both RIF and INH by our methodology. 2691 (73.66%) isolates out of 3653 were Pan-sensitive. Among these isolates, 523 out of 596 (87.75%) were NSP and 2168 out of 3057 (70.92%) were RT.

216 (5.91%) isolates out of 3653 were characterized as HIV-positive TB patients. Among these HIV-positive isolates, 121 out of 596 (20.30%) were NSP and 95 out of 3057 (3.10%) were RT. 1.71% i.e. 9 isolates out of 525 MDR-TB patients (14.37%) were characterized HIV-positive MDR-TB patients (i.e. resistant to both INH and RIF). Among these HIV-positive MDR-TB isolates, 5 out of 596 (0.84%) were NSP and 4 out of 3057 (0.13%) were RT respectively. Thus, no significant contributions of HIV-positive status were found in relation to MDR causation. It has to be noted, that the MDR incidence rate is much higher in male (M) patients than that of Female (F) patients (in case of NSP isolates M:F=1.34:1.00 and in case of RT isolates M:F=1.88: 1.00). In all the cases, resistance rate is greater in RT patients than those in NSP patients. This observation is in agreement with previously reported WHO global statistics.

Mutation patterns in LPA

The molecular mutations conferring the RIF-resistance and INH-resistance were summarized in Table 3. LPA molecular methodology was used for determination of MDR-TB isolates involves scoring mutations in *katG* and *inhA* loci (conferring INH-resistance) as well as in *rpoB* (causing RIF-resistance). In this table, we presented the detailed molecular characterization of the MDR-related mutations.

Of 597 RIF-resistant strains (525 MDR-TB and 72 *rpoB* strains) examined in this study, 68.67% isolates (410 i.e. 369 MDR-TB and 41 *rpoB* strains) had the most frequent molecular mutation in *rpoB* S531L i.e. MUT3. The difference of *rpoB* S531L mutations in MDR-TB strains compared with RIF-monoresistant strains was also not statistically significant (p=0.022). Other *rpoB* mutations (MUT1 i.e. D516V; MUT2A i.e. H526Y and MUT2B i.e. H526D) were also observed with both MDR-TB and RIF-monoresistant isolates. MUT1 is found in 14.48% (76 of 525) in case of MDR-TB and 11.11% (8 of 72) for RIF-monoresistance. This difference of *rpoB* D516V mutations in MDR-TB strains compared with RIF-monoresistant strains was not statistically significant (p=0.965). MUT2A is found in 4.38% (23 of 525) in case of MDR-TB and 12.50% (9 of 72) for RIF-monoresistance. This difference of *rpoB* H526Y mutations in MDR-TB strains compared with RIF mono resistant strains was statistically significant (p=0.003). MUT2B is found in 7.24% (38 of 525) in case of MDR-TB and 2.77% (2 of 72) for RIF-monoresistance. This difference of *rpoB* H526D mutations in MDR-TB strains compared with RIF mono resistant strains was not statistically significant (p=0.154).

The most frequent mutation found in INH resistant strains was *KatG* S315T1 (i.e., MUT1). Of 890 INH-resistant strains (525 MDR-TB and 365 INH-monoresistant strains), MUT1 was found with a frequency of 75.39% (671 i.e. 403 MDR-TB and 268 INH-monoresistant strains) which occurred more commonly in MDR-TB

strains (76.76% i.e., 403 of 525) compared to INH-monoresistant strains (70.14% i.e., 268 of 365). This difference was statistically non-significant (p=0.255). MUT2 (i.e., *KatG* S315T2) is found in 0.76% (4 of 525) in case of MDR-TB and 0.54% (2 of 365) for RIF-monoresistant isolates. This difference of MUT2 mutation in MDR-TB strains compared to INH-monoresistant strains was statistically insignificant (p=0.690).

As reported in the previous studies (7,10,15), almost all *inhA* mutations were found to map in the promoter region (reported to be promoter-up mutations). The *inhA* mutation with the highest frequency was MUT1 [i.e., C(-15)T]. This mutation was found with a frequency of 23.24% (122 of 525) in case of MDR-TB strains and with 17.33% (91 of 365) in INH-monoresistant strains. The difference of MUT1 frequencies between MDR-TB strains compared to INH-monoresistant was statistically insignificant (p=0.560).

MUT2 [i.e., A(-16)G of *inhA*] is found in 0.57% (3 of 525) of MDR-TB isolates and 0.27% (1 of 365) of RIF-monoresistant isolates. This difference of MUT2 mutation between MDR-TB strains and INH-monoresistant strains was statistically insignificant (p=0.509). MUT3A [i.e., T(-8)C of *inhA*] is found in 4.00% (21 of 525) of MDR-TB isolates and 4.38% (16 of 365) of RIF-monoresistant isolates. This difference of MUT3A mutation between MDR-TB strains and INH-monoresistant strains was statistically insignificant (p=0.780). MUT3B [i.e., T(-8)A of *inhA*] is found in 0.38% (2 of 525) of MDR-TB isolates and 0.27% (1 of 365) of RIF-monoresistant isolates. This difference of MUT3B mutation MDR-TB strains and INH-monoresistant strains was statistically insignificant (p=0.778).

Discussion

Accurate determination of drug resistance profile of TB patient isolates is of paramount importance for containment of the global public health challenge posed by the MDR-TB & XDR-TB strains. Additionally, proper and expedited characterization of drug resistance profile of MTB isolates is equally important for cost-controlled administration of anti-TB therapy regimens; this approach controls generation of serious side-effects of second line anti-tuberculosis drugs as well.

Our study was performed in the BSL-3 laboratory of the IRL facility located in the city of Kolkata of the state of West Bengal in Eastern India. In the current study, we reported the results obtained from the LPA tests carried out directly on sputum samples collected from the TB-patient pools including MDR-TB suspects. These laboratory results were compiled for later communication to physicians providing care to the TB-patients. This LPA assay has previously been proven to be highly sensitive (>95%) and specific (>95%) based on comparison with DST assays previously performed in our laboratory and also based on the cross-comparison of LPA results performed in the National Reference Laboratory (NRL), Bengaluru (data not shown).

Our LPA based results from a vast pool of MTB organisms (consisting of 3653 sputum positive isolates) indicated the presence of 2.35% NSP cases and 16.72% RT were found to be MDR-TB isolates. This data matches in general with the global WHO result which estimated 3.9% of NSP cases and 21% RT cases to be MDR-TB [1]. Therefore, we conclude that our MDR rate is comparable to that of the other investigations performed in the rest of the world. We also found that the male patients are more likely to contract the MDR strains than the female patients (1.97: 1.0) which also conforms to the global statistics.

An Ethiopian study showed that among MDR-TB cases who were defaulters in their first-line TB treatment, 62.5% were males (i.e. 1.66: 1.0) [18]. The correlation between being male and contracting MDR-TB could be due to the fact that, males are more prone not to adhere to anti-TB treatment rather than females, thus they have greater risk of developing MDR-TB. From a Chinese study it has also been shown that, Male are more likely to come down with MDR-TB [19]. The male:female (M:F) ratio was found to be 2.7 with 1340 male MDR-TB isolate and 484 female MDR-TB isolates. Similarly, male were shown to be more prone to contract MDR-Tb in other parts of the world also.

Traditionally, TB patients are more likely males than females in most countries [20]. Even the latest WHO statistics show that the male: female ratio of all TB cases is 1.6: 1.0 [1]. This higher rate of TB in men is probably partly due to the fact that women have less access to diagnostic facilities. However, a real epidemiological difference between men and women has been reported both in terms of exposure to infection and susceptibility to disease [20].

Among the cases of patients registered and reported for TB treatment under WHO programs in 2005, the male:female ratio was 1.0:0.7 (20). In a San Francisco based study conducted in the year 2000, the male:female ratio was reported as 2:1 [21]. In surveys conducted in South-East Asia and the Western-Pacific Region, the female:male (F:M) prevalence ratio was found to be less than 0.5 [22].

In Sum, men are more likely to acquire both MDR-TB and the disease tuberculosis in general [15]. Therefore, it is difficult to predict whether any gender-linked factor plays any significant role in generation and propagation of MDR-TB. So, more future studies monitoring male:female ratio related to MDR-TB causation would be necessary to understand any sex-specific factor influencing the occurrence of MDR-TB.

Gender-based differences of MDR-conversion rate were observed in several age groups (Table 2). Male patients generally displayed greater MDR-rate across the board (for example, in age groups of 11-20, 21-30, 31-40, 41-50, 61-70 and 71-80) with the exception of two age-categories 51-60 and 81-90. Again, in age group 81-90, the number of patients is too small to reach any significant conclusion in terms of gender-related propensity of MDR conversion. Therefore, at this point of time, we are unable to interpret this data based on any gender-linked factor. Nevertheless, irrespective of gender-linked consideration, the most significant age-wise MDR-conversion rate is from the age group of 81-90. Here, the overall MDR-conversion rate is extremely high (>42%). A probable cause of this age related increase of MDR-rate can be due to the increased reactivation of tuberculosis disease.

Based on the molecular mutation characterization data presented in Table 3, our observations in terms of the frequencies of different RIF-resistant point mutation are similar to those reported earlier [15]. Among the *ropB* mutations, the MUT3 (*rpoB* S531L) has been observed with the greatest frequency. This data would indicate that the amino acid at the position 531 is quite amenable to a point mutational alteration.

Likewise, in terms of INH-R mutations, the data presented in Table 3 are comparable to earlier reported results [15]. The *katG* MUT1 (*katG* S315T1) is the most commonly occurring mutation. This data would indicate that the amino acid at the position 315 of *KatG* is rather flexible to alteration. Again, in the *inhA* gene, the most frequent mutation has mapped in the promoter region [C(-15)T]; this mutation

is supposed to be a promoter up-mutation [7,15] which generally correlates with a low-level of INH resistance [23].

According to previously reported INH-resistant mutational data, mutations in genes other than *inhA* and *katG* (like *kasA*, *ahpC*, *furA*, etc.) may also result in INH-resistance [15, 24]. Therefore, INH-resistant data communicated in this report is likely to be an underestimation as DST was no longer performed in our laboratory. In future, LPA assays based on these untested loci might be performed for a more complete characterization of INH-resistant isolates from TB patients.

The HIV-positive MDR-rates presented in this study (Table 1) did not show any association of the HIV+ status with increased MDR conversion. In any case, our HIV+ data collection is rather small to be of statistical significance and, thereby, cannot be interpreted meaningfully.

Lastly, this study found a high level of drug resistance in people who have received prior course of anti-TB drugs; this observation resembles previous reports [1]. Repeated anti-tubercular drug courses increase the possibility of resistance including generation of multi-drug resistance among patients with tuberculosis.

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