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Original Article

Type and Frequency of mutations in *katG* and *rpoB* genes in Multi-Drug Resistant Strains of *Mycobacterium Tuberculosis* Complex

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Abstract

Background: Tuberculosis remains a major cause of morbidity and mortality in several parts of world. Genetic basis and mutations in *katG* and *rpoB* genes are responsible for isoniazid and rifampicin resistance in most of the cases of *Mycobacterium tuberculosis*.
Objectives: To determine the mutations in *katG* and *rpoB* genes in confirmed multi-drug resistant tuberculosis isolates and to find the frequency of mutations
Study design, settings and duration: This descriptive study was undertaken in PHRC TB research Centre, Department of Pulmonology, KEMU/Mayo Hospital Lahore. Polymerase chain reaction and genotyping was done at Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore, Lahore from June 2013 to July 2014.
Patients and Methods: A total of 100 acid-fast bacilli smear positive specimens of MDR TB suspects and rifampicin resistant on GeneXpert were collected. Drug susceptibility of isoniazid and rifampicin was carried out by standard drug proportion method. Gene amplification and sequencing was done to detect mutations in *katG* and *rpoB* genes.
Results: A total of the 53% were females and 47% males with male to female ratio of 1:1.1. Mutations in *rpoB* Gene were found to be 98% of rifampicin resistant cases and in *katG* 76.7% of isoniazid resistant cases. Most of the mutations (60%) in *rpoB* Gene were observed on codon 531 while all the mutations in *katG* Gene were observed on codon 315. No novel mutation was found in this study.
Conclusion: Mutation pattern of *rpoB* gene that confers rifampicin resistance is different to a little extent from other national and international studies while pattern is same for *katG* gene that confers isoniazid resistance. No novel mutation was observed in present study.
Key words: Mutations, MDR TB, tuberculosis, *katG* and *rpoB* genes.

Introduction

Tuberculosis (TB) still represents a severe health problem in several parts of the world. Although TB morbidity decreased to some extent in recent years, the situation remains alarming due to a major increase in the incidence and prevalence of multidrug-resistant (MDR) TB.[1] The MDR phenotype is defined as resistance at least to isoniazid and rifampicin the two most effective drugs recommended by the WHO for the first-line treatment of TB with or without resistance to any other first line anti tubercular drugs.[2] Early diagnosis of MDR TB strains is one of the most effective measures for the control of MDR TB.[3]

Conventional methods of detection of drug resistant TB require long time result in delaying proper treatment and contribute to the transmission of drug resistant strains.[4] Resistance to rifampicin has become a useful indicator of possible MDR TB because around 90% rifampicin resistant cases are also resistant to isoniazid.[5] However, this phenomenon does not meet the definition of MDR TB where simultaneous resistance to isoniazid and rifampicin in an isolate is desired.[6] It is estimated that only 5% of patients with MDR-TB are currently detected worldwide due to lack of laboratory facilities in high TB burden countries.[7]

Use of polymerase chain reaction (PCR) amplification and MTB DNA sequencing analysis detects

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Authors Contribution

MKM has done the conceptualization of project. MKM, MS and SR did the data collection. RI has done the literature search. SS did the statistical analysis. Drafting, revision and writing of the manuscript were done by MKM, RI and MA.

mutations in different genes and is predictive of drug resistance with the potential to provide rapid detection of isonoazid, rifampicin and or MDR strains of MTB.[8] Studies have already demonstrated that isoniazid resistance is most frequently associated with specific mutation in *katG* gene (codon 315)[8] in additions however isoniazid resistance has also been found to be associated with two gene locus containing *mabA* and *inhA* in older studies.[9] The mutations in *rpoB* (mostly in 69-bp region) gene are responsible for rifampicin resistance.[10] WHO has recommended adopting the line probe assay that detects these mutations for rapid detection of MDR TB which is expensive hence requires high level of laboratory equipment and commercial kits.[11]

This study was planned to determine the type and site of mutation in *katG* and *rpoB* genes in confirmed MDR-TB isolates and to find the frequency of these mutations.

Patients and Methods

This was a Descriptive study undertaken in PHRC TB research Centre, Department of Pulmonology, KEMU/Mayo Hospital Lahore. PCR and genotyping was done at Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Lahore from June 2013 to July 2014.

The sample size was estimated by using 5% level of significance with expected frequency of MDR among patients having history of treatment 22.12%4 and 8.1% margin of error and thus resulting in total of 100 Ziehl Neelsen (ZN) Smear positive sputum specimens.

Smear positive MDR suspects of both genders and age ≥18 years who were rifampicin resistant on GeneXpert were included in present study. However confirmed MDR cases already taking second line ATT were excluded from the study.

The ethical clearance was taken from institutional review board (IRB). A total of 100 acid-fast bacilli (AFB) Smear positive specimens of MDR-TB suspects who were rifampicin resistant by GeneXpert were collected from out patients and inpatients Department of Chest Medicine, Mayo Hospital Lahore. An informed consent was also obtained from each patient.

Direct and concentrated smears were prepared from clinical specimens. Specimens were treated with 4% NaOH (sodium hydroxide) for decontamination and digestion of clinical specimens. Sterile phosphate buffer PH 6.8 was added to neutralize the effect of NaOH and the samples were concentrated by centrifugation at 3000 rpm for 15 minutes. Supernatant were discarded and sediment was re-suspended in small amount (1-2 ml) of phosphate buffer. MTB culture was inoculated on Lowenstein Jensen (LJ) media by adding one drop on already labeled slants and rest of the sediment was transferred to 2 ml capacity micro-centrifuge tubes. The organisms present in the specimens then were heat deactivated by keeping them at 90C° for 20 minutes and were reserved at –18C° for further processing by molecular method.

The smears were stained with ZN method using 1% carbol fuchsin, 25% sulphuric acid and 0.3% methylene blue. A minimum of 100 oil fields were observed to declare negative smear. Smears were considered positive if it contains at least 3 AFB in observed 100 oil fields for this study. The results were reported according to WHO/International Union of against Tuberculosis and Lung Diseases (IUATLD) recommendations.[12] Drug susceptibility of isoniazid and rifampicin was carried out by standard drug proportion method as described in earlier studies.[4]

Already preserved smear positive decontaminated specimens were thawed and centrifuged in micro-centrifuge tubes at 3000 rpm for 15 minutes at room temperature to concentrate the Mycobacterium tuberculosis bacilli. Supernatant was discarded and a 100µl was transferred to 400 µl Tris-EDTA buffer and heated for 20 min at 80°C to kill the Mycobacterium. Then lysozyme (1 mg/ml) was added to each tube and incubated for 2 hours at 37°C. Then proteinase K (0.2 mg/ml) and 10% SDS (1.1%) were added, vortex and incubated at 65°C for 20 min. During this steps a mixture of CTAB (40 mM) and NaCl (0.1 M) were heated at 65°C and was then added and vortex. When the suspension turned milky, it was then be incubated at 65°C for 10 min. A mixture (750 µl) of chloroform-isoamyl alcohol (24:1) was added, again vortex and then centrifuged at 13,000 rpm in a micro-centrifuge for 5 min at room temperature. The genomic DNA was precipitated with 70% ethanol and re-suspended in 30 µl TE buffer.

For analysis of mutations in drug resistant genes, PCR amplification of target gene and sequencing was done. The primers sequences along with other detail for different genes are listed in Table-1.

Table 1: Primers used for various drug resistant genes amplification.

Gene	*Primer*	*Sequences 5'→3'*	*Product Size (bp)*
rpoB	TR8	TGCACGTCGCGGACCTCCA	157
	TR9	TCGCCGCGATCAAGGAGT	
rpoB	TB-176	CTTCTCCGGGTCGATGTCGTTG	365
	TB-176	CGCGCTTGTCGACGTCAAACTC	
rpoB	TBB-1	ATCACACCGCAGACGTTG	749
	TBB-2	TGCATCACAGTGATGTAGTCG	
katG	TB86	AAACAGCGGCGCTGGATCGT	209
	TB87	GTTGTCCCATTTCGTCGGGG	

The target genes were amplified by preparing 25 µl PCR reaction mixture containing 2.5 µl of one-tenth diluted PCR buffer, 0.5 µl of 10 mMdNTPs, 0.75 µl of 25 mM magnesium chloride solution, 1.25 µl each of the 100 µM primers, 0.125 µl of 2 U AmpliTag gold polymerase (Perkin Elmer, USA), 1.25 µl of DMSO, 2 µl of genomic

DNA, and 15.3 µl of double distilled water. Five µl PCR products were mixed with 1 µl exonuclease and 1µl alkaline phosphatase for purification. The products were then placed in thermal cycler with hot lid off. The cycling parameters were as 30 min at 37°C and 15 min at 80°C. The PCR amplified products were confirmed by running on 1.5 % agarose gel.

PCR products were cleaned by using specific kits (DNA purification kit), after checking the optical density (OD) of DNA, they were subjected for sequencing through Genetic Analyzer for Sequencing: ABI3500. DNA sequencing reaction will be done by using cycle sequencing kit (Beckman Coulter). Sequencing reactions will consist of dH2O, DNA Template (25-100 fmol), Sequencing Primer (1.6pmol/µL) and Dye Terminator Cycle Sequencing Quick Start Master Mix (8.0 µL) to make total volume upto 20 µL. Thermal cycling program was as denaturation at 96C° for 20 seconds, annealing at 50°C for 20 seconds and extension at 60C° for 4 minutes for 30 cycles followed by holding at 4C°. In the cases, that PCR product has not enough intensity for being sequenced it was cloned in the specific vectors such as M13mp18, TA cloning vector or easy vector. All this process was done on commercial basis.

Data analysis was done by using the SPSS. General characteristics of study population are described by using general statistics as mean, median, mod and standard deviation. The frequencies of MDR cases on the basis of drug proportion method and mutations observed in sequencing are presented by using percentage and frequencies.

Results

A total of 100 smear positive and rifampicin resistant on GeneXpert were included; consist of 53% females and 47% males with male to female ratio of 1:1.1. Most of (61%) patients belong to District Lahore and rests were from other districts of Punjab as shown in Table-2.

Characteristics like age, family size, socio-economic status, family history of tuberculosis, history of smoking, education and smear results of study subjects were analyzed and shown in Table-3. Most of the study subjects (87%) lie in the age ≤ 45 years of age, 84% having low socio-economic status and 30/47 (63.8%) males were addicts.

Cough and fever were the most prominent sign and symptoms present in all of the 100% subjects Breathlessness was present in 54% subjects and hemoptysis was noticed in 7% as presented in Table-4.

It was observed that 90% subjects resistant to rifampicin were also resistant to isoniazid. There was significant difference in primary and acquired drug resistance with *p*-value <0.005 as shown in Table-5.

Table 2: District wise distribution of study subjects.

District	*Gender*					
	Female		*Male*		*Total*	
	N	*%*	*N*	*%*	*N*	*%*
Lahore	30	56.6	31	66.0	61	61.0
Sialkot	4	7.5	3	6.4	7	7.0
Sheikhupura	5	9.4	1	2.1	6	6.0
Gujranwala	5	9.4	0	0.0	5	5.0
Okara	3	5.7	1	2.1	4	4.0
Nankana Sahib	0	0.0	4	8.5	4	4.0
Gujrat	0	0.0	3	6.4	3	3.0
Wazir Abad	2	3.8	0	0.0	2	2.0
Mandi Bahauddin	2	3.8	0	0.0	2	2.0
Bahawalpur	0	0.0	2	4.3	2	2.0
Shakargrah	1	1.9	0	0.0	1	1.0
Narowal	0	0.0	1	2.1	1	1.0
Kasur	0	0.0	1	2.1	1	1.0
Faisalabad	1	1.9	0	0.0	1	1.0
Total	53	100	47	100	100	100

Mutations in *rpoB* gene were found to be 98% of rifampicin resistant cases and in *katG* 76.7% of isoniazid resistant cases. Single point mutations in both rpoB and *katG* genes were found in each strain however different types of mutations were found in *rpoB* gene of various strains while only one type of mutation in *katG* gene of all strains was detected. S315L was predominant mutation site in *rpoB* gene and S315T was only site of mutation in *katG* gene of all strains. No novel mutation was found present study as shown in Table-6.

Discussion

MDR TB is an increasing global problem and its burden varies in various areas of world. As study subjects were initially screened with GeneXpert MTB Rif assay and only rifampicin resistant cases were included in present study therefore 100% cases were resistant to rifampicin and 90% were proved as MDR TB cases by drug susceptibility testing by standard drug proportion method on LJ medium in this study however is not comparable with the recent study published in this area which showed lower MDR cases of 17%.[4] Statistically significant difference in primary and acquired MDR TB was observed (p-value <0.05) in the current study which is in agreement with former studies under same settings.[4,13] Reports regarding MDR TB from various areas of Pakistan are not comparable with each other as a high MDR TB of 30% in treated cases with 2% increase from their previous study has been reported in Rawalpindi.[14] Another study from Sindh has also reported 25% MDR TB in treated cases.[15] Much higher (47%) MDR TB was observed in a study from Agha Khan University Karachi showing 10% primary and 69% acquired MDR TB.[16] Higher levels of acquired resistance in this study may be reasoned as physician's error or patient's poor compliance during treatment of susceptible TB.[17]

Table 3: Various characteristics of study subjects.

Characteristics		Gender					
		Female		Male		Total	
		N	%	N	%	N	%
Age	<= 25	21	39.6	19	40.4	40	40.0
	26 - 35	16	30.2	11	23.4	27	27.0
	36 - 45	11	20.8	9	19.1	20	20.0
	46 - 55	2	3.8	2	4.3	4	4.0
	56+	3	5.7	6	12.8	9	9.0
	Mean	31.6 ± 11.7		34.4 ± 13.5		32.9 ± 12.6	
Family size	<= 5	11	20.8	7	14.9	18	18.0
	6 - 7	13	24.5	10	21.3	23	23.0
	8 - 9	13	24.5	12	25.5	25	25.0
	10 - 11	7	13.2	6	12.8	13	13.0
	12+	9	17.0	12	25.5	21	21.0
	Mean	8.3 ± 3.0		9.0 ± 3.2		8.7 ± 3.1	
Socio-economic status	Lower	44	83.0	40	85.1	84	84.0
	Lower Middle	8	15.1	6	12.8	14	14.0
	Upper Middle	1	1.9	1	2.1	2	2.0
	Higher Above	0	0.0	0	0.0	0	0.0
Family history of tuberculosis	Present	36	67.9	34	72.3	70	70.0
	Absent	17	32.1	13	27.7	30	30.0
History of smoking	Present	3	5.7	30	63.8	33	33.0
	Absent	50	94.3	17	36.2	67	67.0
Education	Illiterate	15	28.3	20	42.6	35	35.0
	Primary	25	47.2	18	38.3	43	43.0
	Middle	7	13.2	5	10.6	12	12.0
	Matric	6	11.3	4	8.5	10	10.0
Smear result	Scanty	5	9.4	4	8.5	9	9.0
	1+	22	41.5	17	36.2	39	39.0
	2+	13	24.5	17	36.2	30	30.0
	3+	13	24.5	9	19.1	22	22.0
History of BCG vaccine	Present	53	100.0	44	93.6	97	97.0
	Absent	0	0.0	3	6.4	3	3.0
History of ATT	Present	32	60.4	27	57.4	59	59.0
	Absent	21	39.6	20	42.6	41	41.0
History of contact	Yes	41	77.4	39	83.0	80	80.0
	No	12	22.6	8	17.0	20	20.0

Table 4: Sign and symptoms of study subjects.

Symptoms	Gender					
	Female		Male		Total	
	N	%	N	%	N	%
Fever	53	100.0	47	100.0	100	100.0
Cough	53	100.0	47	100.0	100	100.0
Fatigue	53	100.0	44	93.6	97	97.0
Anorexia	35	66.0	33	70.2	68	68.0
Weight loss	51	96.2	47	100.0	98	98.0
Chest pain	25	47.2	29	61.7	54	54.0
Hemoptysis	2	3.8	5	10.6	7	7.0
Expectoration	52	98.1	45	95.7	97	97.0
Breathlessness	23	43.4	31	66.0	54	54.0

Clinical signs and symptoms are important and were ominously present in study subjects especially cough (100%), fever (100%) and expectoration (97%), weight loss (98%) which are also reported elsewhere.[18]

Mutations in *rpoB* gene are considered to be responsible for majority of rifampicin resistance in MTB.[19] A total of 100 isolates of MTB were resistant to rifampicin by GeneXpert and on LJ medium in present study of which 98% had mutations in their *rpoB* gene. Analysis of these mutations was based on 157 bp region including the 81bp hyper-variable RRDR of *rpoB* gene. All isolates had a single point mutations and highest frequency of mutation was observed in codon 531 (60%) followed by 516 (20%), 526 (8%) 572 (4%) and 582 & 512 (3%).

Table 5: Drug susceptibility pattern of isoniazid and rifampicin.

Drugs		Primary		Acquired		Total	
		N	%	N	%	N	%
Isoniazid	Resistant	33	80.5	57	96.6	90	90.0
	Sensitive	8	19.5	2	3.4	10	10.0
Chi-square = 7.08						*p*-value = 0.008	
Rifampicin	Resistant	41	100.0	59	100.0	100	100.0
	Sensitive	0	0.0	0	0.0	0	0.0
Chi-square = ---						*p*-value = ---	

Majority of isolates showed mutations in codon 531 are consistent with previous study from our country [19] However, contrary findings were shown in a study from Guangxi Zhuang, China showing highest mutations in codon 526 (50%) followed by 531 (35%) and in 516 (8%).[20] Another recent study from china also showed the results similar to the previous study which elaborated 51.2% mutations in codon 526, lesser 10.7% in codon 531 and quite similar 6% in codon 516.[21] Comparable results were also reported from Turkey i.e. codon 531 (52%) and higher rate of mutation on codon 526 (19%).[22] Similarly higher rates of mutations in codon 526 of *rpoB* gene were reported from India (19%), Pakistan (22.5%) and Iran (45.6%) as compared to this study.[19,23,24] Assay used in this study failed to detect 2% of rifampicin resistant isolates while a study using commercially available kit failed to detect 3.7% isolates [25] and results are similar to the present study.

Table 6: Sites of mutation in *rpo*B and *kat*G genes.

Mutations	*Primary*		*Acquired*		*Total*	
	N	*%*	*n*	*%*	*n*	*%*
			rpoB Gene			
S531L	24	58.5	31	52.5	55	55
S531W	3	7.3	2	3.4	5	5
D516L	0	0.0	3	5.1	3	3
D516V	1	2.4	4	6.8	5	5
D516Y	4	9.8	3	5.1	7	7
D516F	2	4.9	3	5.1	5	5
H526Y	1	2.4	4	6.8	5	5
H526C	0	0.0	3	5.1	3	3
S512T	0	0.0	3	5.1	3	3
I572F	3	7.3	1	1.7	4	4
N582N	1	2.4	2	3.4	3	3
No mutation	2	4.9	0	0.0	2	2.0
Total	41	100	59	100.0	100	100.0
			katG Gene			
S315T	26	78.8	43	75.4	69	76.7
No Mutations	7	21.2	14	24.6	21	23.3
Total	33	100.0	57	100.0	90	100.0

It has already been investigated that isoniazid resistance was most frequently associated with specific mutation in *katG* gene and up till 95% of isoniazid resistant strains have been found to contain mutations in codon 315 of *katG*.[8] In present study 90% were phenotypicaly identified as isoniazide resistant using drug proportion method are not in agreement with a study that identified 54.6% isolates.[25] Out of 90 isoniazid resistant isolates 76.7% had mutations in *katG* gene and all the mutations were detected at codon 315 that converted AGC→ACC however not in agreement with another study done by Bostanabad et.al.[8] Results of this study are in concord with a study from Vietnam showing 78% of isoniazid resistant isolates carried a mutated *KatG* codon 315.[26] Lower frequency of mutation on *katG* codon 315 presenting 63% and 66% respectively from various areas of Pakistan is not in agreement with present study.[19,25] Various studies from other countries showed different frequency of mutations on codon 315 of *katG* as study from Singapore showing 63.5% mutations,[27] study from Italy expressed 66.5% mutations [28] and 60.6% mutations from china.[29] Assay used remained unable to detect 23.3% of phenotypically isoniazid resistant strains which is comparable with other study that used a commercially available kit and failed to detect 23.7% of isoniazid resistant cases.[25] As isoniazid resistance has also been reported to be associated with two other gene locus containing *mabA* and *inhA*.[9] It is therefore one of the limitation of the study that these two genes *mabA* and *inhA* were not analyzed for mutations.

In short mutation pattern of rpoB gene that confers rifampicin resistance is different to a little extent from other national and international studies while pattern is same for *katG* gene that confers isoniazid resistance. No novel mutation was observed in present study. Investigation of additional genes like *mabA* and *inhA* are also recomended to completely cover isoniazid resistance.

Most of the mutations occur on codon number 531 of rpoB genes where amino acid change occurs from TCG to TTG or TCG to TGG. Although various single point changes are observed however *rpoB* is specific and mutation in this gene covers 98% rifampicin resistance as shown in present study.

While mutation at codon number 315 where AGC changes to ACC which leads to isoniazid resistance hence covering *katG* gene only. This showed 76.7% resistance to isoniazid.

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