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A RETROSPECTIVE STUDY OF SECOND LINE DRUG RESISTANCE AND THEIR MUTATIONAL PATTERNS IN *MYCOBACTERIUM TUBERCULOSIS* USING GENOTYPE MTBDRSL ASSAY

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ABSTRACT

Background: As a persistent global health problem Pulmonary Tuberculosis is causing immeasurable suffering and wreaking havoc in mankind especially in developing countries.[1,2] A notable challenge in control and management of tuberculosis is the drug resistance to anti Tuberculosis drugs leading to Multi Drug Resistant Tuberculosis (MDR-TB) and Extensively Drug Resistant Tuberculosis (XDR-TB).[3] This is further compounded by the expensive treatment regimens, adverse side effects and toxicity in patients undergoing treatment.[5,6] After the approval by WHO there is a great deal of interest evoked worldwide and in India to use Molecular Genotype susceptibility tests for rapid detection of drug resistance (MDR-TB and XDR-TB) in Pulmonary Tuberculosis.[3] At the present moment the Genotype MTBDRsl assay (hain life science, Germany) is the only available commercial assay to detect Drug Resistance to Second Line anti tuberculosis drugs.[8] This test detects and targets resistance to Fluoroquinolones and second line Injectable drugs Amikacin (Am), Kanamycin (Km) and Capreomycin (Cm). ***Materials and Methods***: This study has been done in IRL-Visakhapatnam to know the XDR cases in Diagnosed cases of MDR and INH mono Resistant pulmonary tuberculosis and to know the pattern of mutations in XDR and Pre-XDR cases. The samples received in the IRL lab. between January 2018 and December 2018 which were drug resistant either MDR-TB/RR-TB or Isonaizid resistant were analysed. ***Results:*** This study conducted has shown that out of the 2038 sample received 1279 samples were Males (62.70%) and Females were 759 (37.20%). The Sex ratio is 2:1. Among the MDR isolates Fluoroquinolone resistance was 15.25%. and second-line Injectable drugs resistance was 0.9% The combined Fluoroquinolone and second line injectable Aminoglycoside resistance was 0.5%. In this study the Genotype MTBDRsl assay has shown a mutation in Codon 94 for Fluroquinolone's resistance. Amongst the mutations in codon 94 the most commonly seen mutation was *gyr-A* MUT3c in 63 samples. In 6 cases we have observed the rare *gyr-A* MUT3d mutation which has not been reported by other such similar studies making it a unique feature. In 29 samples we found *gyr-B* WT1 missing which indicates the presence *gyr-B* mutations in this region. In addition to the above observation, out of the *rrs* mutations for second line injectable drugs we found that a *rrs* MUT1 was seen commonly in 12 cases while *rrs* MUT2 was seen in 11 samples. This study also demonstrated mutations in 5 samples in *eis* region which were detected by the absence in *eis* MUT2.

INTRODUCTION

The Dual Epidemic of Pulmonary Tuberculosis and HIV AIDS remains a Global health concern responsible for a great deal of morbidly and mortality in developing nations.[1,2] A notable challenge in control and management is the drug resistance to anti tuberculosis drugs now referred to as multidrug resistance tuberculosis (MDR-TB) and extensively drug resistance tuberculosis (XDR-TB).[3] The cause for MDR-TB in strains causing Pulmonary Tuberculosis is due to the resistance to the two most potent first line Anti-Tuberculosis drugs Rifampicin and Isoniazid. XDR-TB is due to the strains being resistant to first line drugs rifampicin and isoniazid as well as a fluoroquinolone (FQ) and any one of the second-line injectable drugs (Amikacin, Kanamycin, Capreomycin).[4] These resistant strains (MDR-TB and XDR-TB) are arduous to treat because of adverse side effects, prolonged treatment, and toxicity of the drugs to patients.[5,6] The national TB elimination programme (NTEP) in India emphasizes prompt and early diagnosis of Pulmonary Tuberculosis and Resistance, which are critical to treat the infected patients and also to prevent spread of resistant bacilli in the community.[3,7] The conventional methods of diagnosing MDR-TB and XDR-TB by using sputum samples by solid and liquid culture are not in vogue because of the long turnaround time and it being a

laborious process[7], and often not available in developing countries. These have been replaced by the Molecular Genotype methods. The Genotype Molecular methods are rapid and results are declared in 48 to 72 hrs which has been approved and endorsed by WHO for detection of resistance to first line drugs and second line drugs in Mycobacterium Tuberculosis.[3] The commercially available Genotype MTBDRsl (hain lfe sciences Germany) is currently employed in India[8], which targets and detects resistance to FQs and injectable drugs Am, Km and Cm.

The main mechanisms underlying resistance to first line and second line Anti-Tuberculosis treatment drugs are the spontaneous point mutations in Mycobacterium Tuberculosis.[9] The cause for FQ resistance in M.tb is mainly mutation in the short discrete region *gyr-A* and less commonly in *gyr-B* region collectively known as quinolone resistance region (QRDR).[10] The cause attributed to FQ resistance worldwide is to mutations in *gyr-A* (80%) in the codons 88, 90, 91 and 94.[10] The Am, Km and Cm resistance has been associated with mutations in the 16S rRNA gene (*rrs*) universally in nucleotide positions 1401, 1402, 1484, (87%).[11,12] Additionally, Km, Am and Cm resistance can be caused due to mutations in *eis*, *tlyA* genes respectively. It is now established and proven that mutations conferring resistance vary between Geno groups and geographically.[13,14]

Aim of the study: This study has been undertaken to know the XDR cases in Diagnosed cases of MDR and INH mono Resistant pulmonary tuberculosis. To know the pattern of mutations in XDR and Pre-XDR cases.

Study Settings

This retrospective analysis was carried out in the Intermediate Reference Laboratory (IRL) department of microbiology Andhra medical college, Visakhapatnam. Under national tuberculosis elimination programme of India. This laboratory is a NABL accredited for the genotype molecular and phenotype drug sensitivity of mycobacterium tuberculosis.

Sample collection: all Sputum samples Rifampicin Resistant TB (RR-TB) and Sensitive (RS-TB) cases diagnosed by CBNAAT in the peripheries Districts of AP were referred to IRL-Visakhapatnam as a part of NTEP Programme as laid down in the guidelines.

MATERIAL AND METHODS

A total of 2038 drug resistant isolates (either MDR-TB/RR-TB or Isoniazid resistant) received between January 2018 to December 2018 were analysed. The details regarding rifampicin resistance and isoniazid resistance were acquired from NTEP requisition form. All the samples from rifampicin and Isoniazid or both resistant (tested by the first line LPA genotype MTBDRplus ver, 2.0 hain life sciences or XpertMTB/rif) were subjected to, second line line probe assay (LPA) (genotype MTBDRsl ver2.0 hain life sciences) to detect the additional Fluoroquinolone and Injectable Aminoglycoside resistance. These isolates were from sputum samples received for diagnosis of second line drug resistance to mycobacterium tuberculosis.

The samples were processed by NALC NaOH method, followed by inoculation of 500 µl of processed sample in MGIT tube containing PANTA and growth supplement.[23] These MGIT tubes were placed in the BACTEC MGIT 960 instrument. The positive culture tube from the instrument was identified as *M. tuberculosis* by using SD Bioline MPT 64 Ag kit (Standard Diagnostics, Inc., Republic of Korea). The positive cultures were processed by Genotype MTBDRsl Ver 2.0 assay. The drug resistance characterization was done by second-line LPA (Genotype MTBDRsl Ver 2.0) only, and no phenotypic DST was performed.

The Genotype MTBDRsl Ver 2.0 assay was performed as per the manufacturer's instructions.[24] In brief, 1 ml of positive liquid culture was centrifuged and the pellet was taken for DNA extraction by GenoLyse kit. Before amplification, the kit components AM-A and AM-B were mixed and then the extracted DNA was added and amplified. The hybridization was performed using TwinCubator/GT-Blot and the results were analysed. The strip contains 27 probes to check internal controls, identification of *M. tuberculosis* complex, and drug targets *gyrA*, *gyrB* for FQ and *rrs*, *eis* for SLID. The missing of wild probe and presence of mutant probe are considered as resistant (Figure 1).

Figure 1: Fluoroquinolones and aminoglycosides drug resistance detection by Genotype MTBDRsl Ver 2.0.

RESULTS

Table-1: Samples Distribution of MDR and INH-mono Resistance with respect to gender.

Gender	MDR		INH-MONO		Total	
	Number(N)	Percentage	Number(N)	Percentage	Number(N)	Percentage
Male	536	26.30%	743	36.40%	1279	62.70%
Female	349	17.10%	410	20.10%	759	37.20%

Total of 2038 samples 1279 were males (62.70%) and Female were 759(37.20%) out of the 1279 male patients (26.30%) were MDR samples and INH mono 743(36.40%). Out of the 759 sputum samples received from females 349(17.10%) and INH mono were 410(20.10%). The sex ratio is 2:1.

Table-2: Distribution of MDR and INH-mono Resistance with respect to age group & sex.

Age Group	MDR			INH-MONO		
	M	F	Total	M	F	Total
Below 14 y	4	4	8	1	5	6
15 to 30	116	90	206	137	87	224
31 to 45	212	123	335	268	134	402
46 to 60	148	107	225	249	134	383
61 above	56	25	81	88	50	138
Total	536	349	885	743	410	1153

Frequency distribution of MDR and INH-mono Resistance with respect to age group and sex is shown in table 2. Among MDR and INH-mono Resistance, maximum is in the age group of 31 – 45 years (16.4%) and (19.7%) with mean age of 39.29 and 39.13 years (S.D = 15.93).

Table 3: Results of Genotype MTBDRsl assay.

Sensitive pattern	Study Population N=2038		
	MDR	INH	Total (%)
Sensitive to Fluoroquinolone and SLID	705	1067	1772(86.9%)
Resistances to only Fluoroquinolone	135	50	185(9. %)
Resistances to only SLID (Km,Cm,Am)	11	8	19(0.9%)
XDR Resistance to Fluoroquinolone and SLID	9	2	11(0.5%)
Results invalid	25	26	51(2. %)
Total	885	1153	2038

Table-3 shows sensitivity pattern of MDR-TB and INH mono resistance detected by Genotype MTBDRsl directly from sputum and indirectly from culture. Out of 2038 clinical specimens, 1772 (86.9%) were susceptible to both Fluoroquinolone & Second Line Injectable Drugs, 11(0.5%) were resistant to both Fluoroquinolone & Second Line Injectable Drugs (ie. XDR). 185(9%) resistant to only Fluoroquinolone and 19(0.9%) resistant to Second Line Injectable Drugs only. The assay has shown 2% results are invalid which is within acceptable limits as per the kit insert.

Table-4: Pattern of gene mutations in M. Tuberculosis from clinical specimens using Genotype MTBDRsl assay.

Fluroqunulones				SLID		Low level kanamycine		N (%)		
gyr-A	Result	*gyr*-B	Result	*rrs*	Result	*eis*	Result	TOTAL	MDR	INH mono
WT	S	WT	S	WT	S	WT	S	1772	705	1067
ΔWT1	R	ΔWT1	R	WT	S	WT	S	1	0	1
ΔWT1	R	WT	S	WT	S	WT	S	2	2	0
ΔWT1,2	R	WT	S	WT	S	WT	S	1	1	0
ΔWT1,2,3	R	WT	S	WT	S	WT	S	1	1	0
ΔWT2	R	WT	S	WT	S	WT	S	5	3	2
ΔWT2,3	R	WT	S	WT	S	WT	S	4	4	0
ΔWT3	R	WT	S	ΔWT M(A1401G)	R	WT	S	1	1	0

ΔWT3	R	WT	S	WT	S	WT	S	9	7	2
M1(A90V)	R	WT	S	WT	S	WT	S	43	32	11
M1(A90V)	R	ΔWT1	R	ΔWT M(A1401G)	R	WT	S	1	1	0
M1(A90V)	R	ΔWT1	R	WT	S	ΔWT2	R	1	1	0
M1,3B (A90V,D94N+Y)	R	WT	S	WT	S	WT	S	1	0	1
M2(S91P)	R	WT	S	WT	S	WT	S	3	3	0
M2,3C (S91P,D94G)	R	WT	S	WT	S	WT	S	1	1	0
M3A(D94A)	R	WT	S	WT	S	WT	S	6	5	1
M3A(D94A)	R	ΔWT1	R	WT	S	WT	S	1	1	0
M3B(D94N+Y)	R	WT	S	ΔWT M(A1401G)	R	ΔWT2	R	1	1	0
M3B(D94N+Y)	R	WT	S	M2 (G1484T)	R	WT	S	1	1	0
M3B(D94N+Y)	R	WT	S	WT	S	WT	S	18	16	2
M3B,3C (D94N+Y,D94G)	R	WT	S	WT	S	WT	S	2	1	1
M3C(D94G)	R	WT	S	ΔWT M(A1401G)	R	WT	S	2	1	1
M3C(D94G)	R	WT	S	WT	S	ΔWT2	R	3	3	0
M3C(D94G)	R	WT	S	WT	S	WT	S	55	43	12
M3D(D94H)	R	WT	S	WT	S	WT	S	6	4	2
WT	S	ΔWT1	R	M2 (G1484T)	R	WT	S	1	0	1
WT	S	ΔWT1	R	WT	S	WT	S	24	10	14
WT	S	M1 (N538D)	R	WT	S	WT	S	1	0	1
WT	S	M2 (E540V)	R	WT	S	WT	S	1	1	0
WT	S	WT	S	ΔWT M(A1401G)	R	WT	S	6	5	1
WT	S	WT	S	ΔWT1+2	R	WT	S	1	0	1
WT	S	WT	S	M2 (G1484T)	R	WT	S	9	5	4
WT	S	WT	S	WT	S	ΔWT1	R	1	0	1
WT	S	WT	S	WT	S	ΔWT3	R	2	1	1
NA		NA		NA		NA		51	25	26

Abbreviations: ΔWT – missing wild-type probe; WT – all wild-type probes present Mutation pattern produced by Genotype MTBDRsl assay are displayed in table 4.

All wild-type probes (WT) gave a positive signal & all mutation probe (M) reacted negatively in 1772 (86.9%). hence are sensitive to Fluoroquinolone & Second Line Injectable Drugs.

Mutations conferring resistance to either Fluoroquinolone & Second Line Injectable Drugs were detected in 215/2038 (10.5%) of samples analysed.

Among 196 Fluoroquinolone resistant isolates detected by Genotype MTBDRsl assay, *gyrA* mutations occurred in 169 (86.2%) & *gyrB* mutation occurred in 31 isolates (15.8%). Four of the 169 strains with a *gyrA* resistance had an additional resistance in the *gyrB* promoter region.

The most frequently observed *gyrA* mutation was *gyrA* D94G (in 60/169 strains, 35.5%) and A90V (in 46/169 strains, 27.2%) followed by missing WT ie. ΔWT 24 (14.2%) in our study.

In our study Out of 31 *gyrB* resistance, 2 strains with a mutation in the *gyrB* promoter region had a *gyrB* N538D and E540V mutation. followed by missing WT ie. ΔWT 29, those 29 strains 3 were *gyrA* (2 were A90V and 1 were D94A), mutation found only ΔWT were 15.3%.

The most commonly observed mutation in our study for Fluoroquinolone resistance in the *gyrA* was in the D94G region (35.5%).

Fluoroquinolone resistant isolates revealed by negative hybridization results with wild-type probes was 29.5%.

Among the 23 Second Line Injectable Drugs resistant isolates detected by this test, the frequency of *rrs* mutations was 11 in A1401G (47.8%), 11 in G1484T (47.8%) & 1 missing WT8 ie. ΔWT8 (4.3%).

Hence A1401G and G1484T is the most frequent type of mutation responsible for Second Line Injectable Drugs resistance in our study.

4.3% of Second Line Injectable Drugs resistant isolates were detected by negative hybridisation results with wild type probes.

8 were Low level kanamycin resistant isolates detected by the frequency of *eis* gene revealed by negative hybridization results with wild-type probes.

DISCUSSION

The initiation of correct regimen for the treatment of second line drug resistance depends on its rapid and early diagnosis. The genotype mdrtbsl assay by hain lifesciences has been endorsed by WHO for this purpose and also implemented by ntep programme in india. In this study, we analyse all the samples which were referred to IRL Visakhapatnam to know the XDR cases in Diagnosed cases of MDR and INH mono Resistant pulmonary tuberculosis and to know the pattern of mutations in XDR and Pre XDR cases under programmatic condition of AP.

In the study Genotype MTBDRsl assay to detect second-line drug resistance and patterns of mutations as endorsed by WHO.

This study conducted in the lab has shown That out of 2038 samples 1279 were males (62.70%) and Female were 759(37.20%). The sex ratio is 2:1.

In the male patients in samples, we received our study has shown that INH mono resistant is 36.40% when compare to MDR which is 26.30%.

In females' patients in samples, we received 20.10% were INH mono resistant and 17.10% were MDR.

The total FQ resistance was 15.25% among the MDR isolates, which is similar to Studies by Ho et al.[15,16] which has shown FQ resistance in MDR patients ranging from 1 to 22%. The overall SLID resistance was 0.9%, which is less than that from other regions of India.[17] Both the FQ and SLID resistance were detected in 0.5% in isolates, which is lower than reported prevalence of XDR TB worldwide.[18] In our study, we also included the isoniazid mono-resistant isolates and found that the FQ resistance in was 4.3%. The meta-analysis done by Ho et al.[19] had shown the prevalence of FQ resistance to be 0–4.4% among non-MDR TB patients [19]. The FQ resistance was also noted in newly diagnosed MDR/RR TB cases, which might be due to the transmission of the drug-resistant strains.

The mutation that was most frequently detected by Genotype MTBDRsl in FQ-resistant isolates was a change at codon 94. In our study we have observed that among codon 94 mutations, the most important mutation was in *gyrA* MUT3C in 63 cases, which is half of what was reported in the studies from South Africa, China, and parts of India.[20,21,22] We observed the presence of a rare *gyrA* MUT3D mutation in six cases, which has not been reported in most of the studies, including the recent study from China.[16] This study has also shown the *gyrB* WT1 missing in 29 cases, indicating the presence of *gyrB* mutations in this region of Visakhapatnam, Andhrapredesh.

Among the *rrs* mutations for the injectable drugs, the most prevalent was *rrs* MUT1 found in 12 cases, while *rrs*MUT2 was present in 11 cases. These observations are comparable to the previous studies from India and South Africa.[20,21] We also found the mutations in *eis* region and the most common was *eis*MUT2 absent in 5 cases.

The limitation of this analysis lies in the absence of phenotypic DST and sequencing data for confirmation of different drug-resistant related mutations. The tuberculosis control program in this region is impaired by Circulating Pre-XDR cases and XDR case, which are increasing alarmingly day to day.

CONCLUSION

The WHO-endorsed rapid method for detection of second-line drug resistance, Genotype MTBDRsl assay, is also able to detect mutations with a short turnaround time, thus enabling early and precise implementation of treatment regimens and therapy to the ailing needy patients.

LPAs are an efficient and reliable rapid molecular DST assay which are useful for screening of MDR and XDR-TB. Especially in high burden countries like India, which will reduce transmission rates, morbidity and improve treatment outcomes in patients.

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