Nudrat *et al. J Pure Appl Microbiol,* **13**(**2**), 1079-1085 | June 2019
Article 5582 | https://dx.doi.org/10.22207/JPAM.13.2.46

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Importance of Detection of Isoniazid and Rifampicin Mono Drug Resistance and Determining Rate of MDR-TB in Smear Positive Sputum Samples from a Tertiary Care Hospital of West U.P. India

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Abstract

Anti-tubercular therapy is one of the effective strategies used to control tuberculosis, so, a planned and accurate treatment regimen is of utmost importance, but number of cases are being treated as MDR on the basis of rifampicin mono resistance. As reported earlier in various studies from India, prevalence rate of Multi Drug Resistant-Tuberculosis (MDR-TB) vary from region to region. Therefore, we set out to determine rate of MDR-TB, Isoniazid and Rifampicin Mono resistance and common mutation pattern associated with them from our area using GenoType MTBDR *plus* assay in order to provide better patient care and reduce rate of MDR-TB. This was a Cross-sectional study comprising of 1100 sputum samples collected from DOTS Centre and processed by ZN staining and LPA. Out of 1100 sputum samples, 203 were smear positive. In 203, 193 were detected as positive for MTBC. Rate of MDR was found 12.8% and rifampicin and isoniazid mono-resistance was 6.4% and 8.3% respectively. Commonest mutation pattern seen was S531L in rifampicin and S315T1 in isoniazid. Association between treatment history and resistance pattern was found to be statistically significant. We found there is a high rate of INH mono resistance which was not being detected till now from this area and we also found, there is unrelated risk of isoniazid and rifampicin mono-resistance so, inference of MDR based on RIF mono- resistance is also an inaccurate strategy to manage patients and drug sensitivity should be performed for both first line drug before stating MDR.

Keywords: ***InhA, katG*, Line probe assay, Polymerase chain reaction, *rpoB.***

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(Received: 22 March 2019; accepted: 20 May 2019)

Citation: Sana Nudrat, Umar Farooq and Mazhar Maqsood, Importance of Detection of Isoniazid and Rifampicin Mono Drug Resistance and Determining Rate of MDR-TB in Smear Positive Sputum Samples from a Tertiary Care Hospital of West U.P. India, *J Pure Appl Microbiol*., 2019; **13**(**2**): 1079-1085. doi: 10.22207/JPAM.13.2.46

INTRODUCTION

Tuberculosis remains as one of the most common infectious disease in developing countries still in 21st century[1]. Tuberculosis is an important international health problem and this issue has become even more prominent as a result of increasing number of drug resistant strains[2]. Anti-tubercular therapy is one of the effective strategies used to control tuberculosis. First line drugs including rifampicin, isoniazid, ethambutol, pyrazinamide and streptomycin are the preferred choice for TB control. Recently MDR – TB strains has become a serious public health problem in developing countries and has consistently been contributing to increased annual TB incidence rates[3]. Resistant to at least isoniazid and rifampicin is of great concern because it requires the use of second line drugs that are difficult to procure and are much more toxic and expensive than the first line regime. Based on a survey, among all *M. tuberculosis* isolates tested for drug susceptibility, 10.9% were resistant to one anti – TB drug and 6.7% were resistant to both isoniazid and rifampicin. It has been proved that patients infected with strains resistant to rifampicin will experience a higher failure rate with short course 6 months chemotherapy. Globally an estimated 3.6% of new cases and 20.2% of previously treated cases are due to MDR – TB[4].

Rifampicin is an effective drug against *Mycobacterium tuberculosis complex*, which interferes with transcription by the DNA dependent RNA polymerase. In most cases resistance to rifampicin is linked with mutation within 81bp hypervariable or hot spot region of rifampicin resistance determining region (RRDR) of the *rpoB* gene. Detection of *rpoB* gene mutation is considered a surrogative marker for MDR – TB detection and can be used as a tool in MDR – TB diagnostics. In Isoniazid, resistance is associated with mutation in *inhA* (position -15 and -8 in the *inhA* promoter sequence), *KatG* (codon 315)[5].

Mutation in the 81bp RRDR of the *rpoB* gene are found in about 96% of rifampicin resistant in *M. tuberculosis.* Mutation at codon 516, 526 and 531 are among the most frequently mutation in rifampicin resistant strains. For isoniazid resistant clinical isolates mutation within the *katG* gene occur most frequently between codon 138 and 328, particularly at codon 315, accounting for 50-95%. Mutation at position -15 and -8 in the *inhA* regulatory region is 6 – 43%[6].

The World Health Organization (WHO) recommended a new policy to use Line Probe Assay (LPA) for rapid screening of patients at risk of MDR – TB in 2008. Geno Type MTBDR*plus* assay (Hain Life Sciences) targets the *rpoB* gene (coding for beta subunit of RNA polymerase), *katG* coding for Catalase peroxidise) and promoter region of *inhA* (coding for NADH enoyl ACP reductase) gene in both culture isolates and clinical samples. Addition to rifampicin resistance (*rpoB* gene), MTBDR*plus* assay aids in the detection of high level and low level resistance to isoniazid via *katG* gene and *inhA* gene respectively[7,8].

As reported in various studies from India, prevalence rate of MDR-TB vary from region to region. Therefore, we set out to determine rate of MDR-TB, mono drug resistance and common mutation pattern from our area using GenoType MTBDR*plus* assay.

MATERIALS AND METHODS

Study design and setting

This was a laboratory based cross sectional study which was carried out in molecular section of Microbiology Department, Teerthankar Mahaveer Medical College and Research Centre, Moradabad, Uttar Pradesh, India.

Specimen Collection

1100 sputum samples were collected from the patients in a clean sterile universal containers attending DOTS centre of Teerthankar Mahaveer Hospital. These samples were transported to Microbiology laboratory according to international standards of WHO recommendation for transport of biological substances.

Sputum Microscopy

These samples were processed for smear microscopy by Z-N staining. 203 samples were smear positive.

Decontamination

Smear positive sputum specimens were processed for decontamination by mixing N-acetyl-L-cysteine and NaOH in the specimen and incubate for 15 min. After that, phosphate buffer was added to the specimen and centrifugation was done for 15 min at 3000 g. Then, discard the supernatant and re-suspend the pellet in 1 ml phosphate buffer.[9]

DNA extraction

500µl of the decontaminated specimen was processed in the micro centrifuge (13000rpm for 15 min at room temp.) The supernatant was discarded and the pellet was re-suspended in 100µl of distilled water and then inactivates the bacteria by incubating in a heating block for 20 minutes at 95°C. After that cells were sonicated in an ultrasonic bath for 15 minutes and centrifuge for 5 minutes at 13000rpm.[9]

DNA Amplification

Amplification was performed by combining 35µl of primer nucleotide mix (PNM A) containing buffer, nucleotides, Taq polymerase and 10µl of primer nucleotide mix (PNM B) containg salt, specific primers, dye. 5µl extracted DNA was mixed in the master mixture (A and B). After that, this mixture was kept in the Thermocycler for the amplification of the bacterial DNA.

Amplification Cycle

15min	95°C	1 cycle
30sec	95°C	
2min	65°C	20 cycles
25sec	95°C	
40sec	50°C	
40sec	70°C	30 cycles
8min	70°C	1 cycle

Genotype MTBDRplus (ver 2.0)

Hybridization was performed manually using Twin incubater / shaking water bath at 45°C as per manufacturer's instructions.[8]

Observation and Results

During the study period July 2015 to June 2017, out of 1100 sputum samples processed for Z-N staining, 203(18.4%). samples were positive for AFB. *M. tuberculosis* complex MTBC was positive in 193 cases out of 203 and 10 samples (4.9%) were detected as Non Tuberculous Mycobacterium (NTM). All 193 *M. tuberculosis* isolates were processed for drug sensitivity by PCR and Line Probe Assay. Out of 193, 26 (12.8%) were MDR, 13 (6.4%) were rifampicin mono-resistant, 17 (8.3%) were isoniazid mono-resistant and 137 (67.4%) were sensitive to both drugs rifampicin and isoniazid as shown in Table 1.

Table 1. Distribution of drug sensitivity pattern in patients

Drug Sensitivity pattern	Number of Samples
MDR	26 (12.8%)
Rifampicin mono-resistant	13 (6.4%)
Isoniazid mono-resistant	17 (8.3%)
Sensitive to both drugs	137 (67.4%)
NTM	10 (4.9%)

In this study, all smear positive cases are divided in 2 categories. 59 cases were in category 1 and 134 were in category 2. Patients in category 1 had not taken anti-tubular drugs previously, i.e; they all were new cases. In category 2 all 134 patients had previously received anti-tubular drugs. Category 2 patients were further classified as defaulter i.e., those who left treatment in between the course of DOTS therapy. Failure cases who remained smear positive after 2 months of treatment and relapse cases were those who turned smear positive again after successful full term DOTS therapy, as shown in Table 2.

In our study a higher prevalence rate of MDR, rifampicin and isoniazid mono-resistance was found among males i.e. 9.3%, 4.4% and 5.9% respectively, and majority of patients were in age group of 26-35 years followed by 36-45 and 46-55 years age group, but neither age nor sex was significantly associated with MDR or mono-resistant status. (p value >0.05) as shown in Table 3 and Table 4.

Table 2. Category wise distribution

Category	No. of patients		Total no
	Male	Female	patients
1	44(21.6%)	15(7.3%)	59(29.0%)
2	96(47.2%)	38(18.7%)	134(66.0%)
	140	53	193

We also found a higher rate of MDR, RIF and INH mono-resistance among defaulter category, 53.8%, 46.1% and 47.0% respectively and sensitivity to both first line drugs was seen highest in new patient category (39.4%). p value calculated was <.05 which shows there is significant association between treatment history of patient and drug resistance , also by calculating OR it was found that there are 7.03 times more chances of MDR-TB in defaulter cases as compared to new cases, so it is a significant risk factor as shown in Table 5.

Table 3. Sex wise distribution pattern among different drug resistant cases

	MDR	χ^2, df, P value	RIF mono resistant	χ^2, df, P value	INH mono resistant	χ^2, df, P value	Sensitive to both drugs	χ^2, df, P value
Male	19(9.3%)	0.01, 1, 0.89	09(4.4%)	0.05, 1, 0.81	12(5.9%)	0.01, 1, 0.89	99(48.7%)	0.01, 1, 0.906
Female	07(3.4%)		04(1.9%)		05(2.4%)		38(18.7%)	
Total	26		13		17		137	

Frequency of gene mutation associated with resistance to rifampicin (*rpoB*) and isoniazid (*katG* or *inhA*) are shown in Table 6. Out of 26 MDR cases, for *rpoB* gene WT 2 band is absent in 3 cases at 510-513 gene region and mutation L511P is present. In 6 cases WT 3,4 band is absent at 513-519 gene region and D516V mutation is present. 16 cases, WT 8 band is absent at 530-533 gene region and in 12 cases MUT3 band is present with S531L mutation. In 2 cases MUT2B band is present at 526-529 gene region and mutation H526D is present. In *katG* gene WT band is absent in 14 cases at 315 gene region and MUT1 band is present in 18 cases at with S315T1 mutation. In *inhA* gene WT1 band is absent in 6 case at -15/-16 gene region and MUT1 band is present in 3 cases at -15 gene region and C15T mutation is present. In only 1 case WT2 band is absent at -8 gene region.

In 13 RIF monoresistance cases, in 1 case WT2 band is absent at 510-513 gene region and L511P mutation is present. In 1 case WT4,5 band is absent at 516-522 gene region and mutation

Table 4. Age wise distribution of patients

Age Group	MDR (%)	RIF Mono-resistant	INH Mono-resistant	Sensitive to Both Drugs	No.of Pt in particular age group	χ^2 df, P value
15-25	01	01	01	17	20	6.646,
26-35	06	02	05	34	47	15, 0.96
36-45	08	03	03	25	39	
46-55	05	03	03	19	30	
56-65	03	01	02	19	25	
66 and above	03	03	03	23	32	
Total	26	13	17	137	193	

Table 5. Rifampicin and Isoniazid resistance pattern in relation to tuberculosis treatment history

Drug resistant pattern	Category	MDR	RIF mono resistant	INH mono resistant	Sensitive to both drugs	χ^2, df, p value
New (n=59)	1	02 (7.6%)	02 (15.3%)	01 (5.8%)	54 (39.4%)	
Failure (n=25)	2	03 (11.5%)	03 (23.0%)	05 (29.4%)	14 (10.2%)	22.567, 9, 0.0072
Relapse (n=38)		07 (26.9%)	02 (15.3%)	03 (17.6%)	26 (18.9%)	
Defaulter (n=71)		14 (53.8%)	06 (46.1%)	08 (47.0%)	43 (31.3%)	
Total (n=193)		26	13	17	137	$p < .05$

Table 6. Frequency of gene mutation associated with resistance to rifampicin (*rpoB*) and isoniazid (*katG* or *inhA*)

Gene		Band	Gene region / Mutation	MDR	RIF resistance	INH resistance
rpoB	1	WT 1	506-509			
	2	WT 2	510-513	03	01	
	3	WT 3,4	513-519	06		
	4	WT 4,5	516-522		01	
	5	WT 5,6	518-525			
	6	WT 7	526-529			
	7	WT 8	530-533	16	09	
	8	MUT 1	D516V			
	9	MUT 2A	H526Y		01	
	10	MUT 2B	H526D	02	02	
	11	MUT 3	S531L	12	03	
katG	12	WT	315	14		09
	13	MUT1	S315T1	18		14
	14	MUT2	S315T2			
inhA	15	WT1	-15 / -16	06		02
	16	WT2	-8	01		02
	17	MUT1	C15T	03		01
	18	MUT2	A16G			
	19	MUT3A	T8C			
	20	MUT3B	T8A			

N518L is present. In 9 cases WT8 band is absent at 530-533 gene region and MUT3 band is present in 3 cases with S531L mutation. In 1 and 2 case, MUT2A and MUT2B band is present at 526-529 gene region and mutation H526Y and H524D is present.

In 17 INH monoresistance cases, for *katG* gene, WT band is absent in 9 cases at 315 gene region and MUT1 band is present in 14 cases with mutation S315T1. In *inhA* gene, WT1 band is absent at -15/-16 gene region and MUT1 band is present in 1 case with C15T mutation. In 1 case WT2 band is absent at -8 gene region.

Statistical Analysis

The collected data was entered in Microsoft excel and analysed using SPSS version 21 (SPSS Inc, Chicago, IL, USA) software was used for making proportional and analysing table. Chi square test was used to determine the association between different variables. P value < 0.05 was considered statistically significant.

DISCUSSION

In this study, it was observed that the rate of TB infection was found to be more in male and the male to female ratio of 2.6:1. Similarly, high male to female ratio was reported by Singhal *et al*[9] and Rao[10]. Although association was found statistically insignificant and could be because males are exposed more to risk factors as compared to females, Age wise distribution of patients also reviled fruitful observations which showed out of 203 more than 50% of patients belong to 26-55 years age group similar findings were reported by Rao[10] WHO also reports that TB mostly affects adults in their most productive years.[11] These observations may have strong implication in TB control strategies.

In present study, MDR TB rate found is 12.8% which is comparatively higher to previously reported by some authors from India like 4.7% reported by Gupta *et a*l,[12] from Lucknow in 2013; 4.5% reported by Malhotra *et al*.[13] from Jaipur in 2002 whereas it is quite low when compared few other studies, such as 14.6% reported by Thakur *et al,*[14] from Solan H.P. in 2015; 21.0% reported by Ahmad *et al*.[15] from Aligarh in 2017 which indicates rate of MDR-TB vary from region to region. In this study moderate rate of MDR TB was observed as only smear positive patients were included in this study whereas studies which reported lower rates included all smear positive and negative cases

and studies which reported higher rates included exclusively MDR-TB suspected cases.

Simultaneously, rate of RIF and INH mono-resistance found in this study was 6.4% and 8.3% respectively, while other studies from India reported RIF mono-resistance rate of 5.8%, 19.4%, 5.4%, 8.8% from Aligarh[15], Solan[14], Varanasi[16], and Delhi[17] respectively and INH mono resistance from same studies were reported as 9.2%, 20.35, 6.3%, and 8.5%. TB control programmes generally focus on MDR-TB because these strains are difficult to treat and cause much morbidity and mortality. Many drug resistant surveys have shown that mono and poly resistant TB are actually more common than MDR TB[14-17]. we also found that rate of mono resistance was more as compared to rate of MDR TB (14.7% and 12.8% respectively). Many of these cases contribute towards amplification of resistance and eventually lead to MDR if they are not properly managed.

In this study it was also observed that prevalence rate of MDR TB is quite high in defaulter, relapse and failure cases as compared to new cases. Prevalence rate for defaulter cases of MDR was 53.8%, for relapse cases it was 26.9% and 11.5% for failure cases which is quite high as compared to prevalence rate of MDR in new cases which was 7.6%. These findings support previously reported findings of Thakur *et al.*[14] Malhotra *et al*.[13] Ahmad *et al.*[15] and Talesse *et al*.[18] statistically a significant association was found between treatment history and drug resistance ($p<0.05$) so it can be said treatment failure or defaulter is significantly associated with drug resistance in TB cases.

Thakur C *et al.*[14] in 2015 reported most common mutation is associated with *rpoB* 530-533 region mostly S531L mutation (62.2%). We also found S531L mutation as the most common mutation associated with rifampicin resistance (61.5%). This resistance was also most common in both MDR cases and rifampicin mono-resistance cases. Similar finding were reported by Hirani *et al.*[19] and Sharma *et al.*[20] Most common mutation associated with isoniazid mono-resistance was S315T1 at region 315. Similar findings were reported by Swaminathan *et al.*[21] and Meaza *et al.*[22]

CONCLUSION

The author found that there is high rate of INH mono resistance which was unrelated to rifampicin mono-resistance so, inference of MDR based on RIF mono- resistance may be an inaccurate strategy to manage patients. Many of the Mono resistant cases contribute towards amplification of resistance and eventually lead to MDR if they are not managed properly, these cases often remain undiagnosed in resource limited settings and also DST is recommended only for that group of patients those are at high risk of developing MDR. Correct treatment of mono drug resistant TB can prevent development of MDR-TB. All TB patients infected with rifampicin mono resistant strains should be treated using a full MDR TB regimen with isoniazid being added in the regimen until DST results to isoniazid are available. If DST of isoniazid shows susceptibility it can be continued in the MDR-TB regimen. Whereas, patients infected with isoniazid mono resistant strains can be treated with rifampicin along with ethambutol and pyrazinamide with caution and with monitoring for possible rifampicin amplification. Performing rapid DST to isoniazid and rifampicin with LPA at the start of treatment would help identify many more cases of mono drug resistance and thus providing appropriate treatment and controlling spread of MDR-TB.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. Umme Afifa for help in statistical analysis. SN also acknowledges support of Shivendra Mohan for help in sample processing and data collection.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

SN performed the tests, collected data, done data analysis and wrote the manuscript. UF guided the study and reviewed the manuscript. MM provided the sample and reviewed the manuscript. SN, UF and MM approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

The study protocol was carefully reviewed and approved by the Institutional Ethics Committee of the Teerthankar Mahaveer Medical College and Research Centre.

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