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RESEARCH ARTICLE
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DETECTION OF IMPORTANCE OF RIFAMPICIN AND ISONIAZID RESISTANCE AND DETERMINE RATE OF MDR-TB IN POSITIVE SMEAR SPUTUM SAMPLES FROM A TERTIARY CARE HOSPITAL OF M.P

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Abstract
One of the most effective strategies used to control tuberculosis is anti-tubercular therapy. In india various studies are reported earlier, the prevalence rate of MDR-TB very from region to region. Therefore , we determine rate of multi drug resistant –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 Multi Drug Resistance –TB. This was a cross sectional study comprising of 800 sputum samples collected from DOTS center and processed by ZN staining and LPA. out of 800 sputum sample, 168 were smear positive . rate of MDR was found 9.88%. commonest mutation pattern seen was S531L in rifampicin and S315T1 in isoniazid. 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.

Kewords: InhA, katG, rpoB, LPA, PCR.

INTRODUCTION

TB is known since the very beginning of civilization and till date it is considered a huge health problem specially in developing nations. Even in 21" Century[1] millions of people fall ill due to TB and according to WHO statistics in the year 2015 TB was one of the top 10 infection causes of deaths worldwide leaving behind number of deaths HIV and caused by these facts become more fearful after knowing that Tuberculosis can be fully cured with proper treatment and timely diagnosis.[2] Tuberculosis is an infectious disease caused mainly by Mycobacterium tuberculosis. Clinically it is of two types Pulmonary and Extra pulmonary. On the basis microbiological investigation pulmonary TB can also be categorized as sputum positive and sputum negative.[3]

Multi drug resistant tuberculosis (MDR-TB) is defined as "TB caused by *M. tuberculosis* complex resistant to rifampicin and isoniazid in vitro, without resistance to any other first line anti-tubercular drag". WHO in 2016 estimated that there were approximately 6 lakh rifampicin mono resistant cases which is considered as most effective anti-tubercular and out of these mono resistant cases [4,5] lakh were simultaneously resistant to isoniazid le, they were MDR and about 6.2% of MDR-TB patients had XDR-TB. According to a report by Glaziou P. et al significant load of MDR-TB falls largely on 3 countries viz, China, India and Russia. These countries have nearly half of MDR cases as compared to rest of world.[6,7]

specific mutation at S315T region in katG gene coding for Catalase peroxidise and/or mutation C15T, AI6G, T8A and T8C promoter region of inhA gene coding for nicotinamide adenine dinucleotide phosphate-oshlase entyl-acyl carnier protein reductase[8]. These all mutations are visible on the nitrocellulose membrane in form of absence of wide type band and/or absence or presence of specific mutations.[9] Mutation in katG gene is responsible for high level isoniazid resistance and mutation in katG gene is responsible for low level isoniazid resistance Mutation in katG gene is significantly associated with pre-treatment and increase the rate of resistance to streptomycin, rifampicin and ethambutol but inhA promoter region mutations is only associated with resistance to streptomycin.[10]

According to WHO data approximately 2 to 3 million people die because of tuberculosis every year i.e. about 5500 persons/day and 95% to 98% of these deaths occur in developing countries like ours. These figures seems more dreadful as they also report that every second someone gets newly infected with tuberculosis and every 10 second a death occurs due to tuberculosis, worldwide. Overall 75% of world population is currently infected with *Mycobacterium tuberculosis* out of which only 5% to 10% show sign and symptoms. Further WHO also states that in 2016, 1.7 million died and approximately 10.4 million become sick due to tuberculosis.[3,4,5]

MATERIALS AND METHODS

Study design

This was a laboratory based cross sectional study which was carried out in molecular laboratory of Microbiology Department, Index Medical College and Research Centre, Indore, MP

Specimen Collection

800 sputum samples were collected from the patients in a sterile universal containers attending DOTS centre of Index 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. 168 samples were smear positive.

Decontamination

Smear positive sputum specimen 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 pallet in 1ml phosphate buffer.[11]

DNA extraction

500ul 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 100ul of distilled water and then inactivates the bacteria by incubating in a heating block for 20 minutes at 95 degree

C. After that cell were sonicated in an ultrasonic bath for 15 minutes and centrifuge for 5 minutes at 13000rpm.[11]

DNA Amplification

Amplification was performed by combining 35ul of primer nucleotide mix (PNM A) containing buffer, nucleotides, Taq polymerase and 10ul of primer nucleotide mix (PNM B) containg salt, specific primers, dye. 5ul 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	1cycle
30sec	95°C	
2min	65°C	20cycle
25sec	95°C	
40sec	50°C	
40sec	70°C	30cycle
8min	70°C	1cycle

Genotype MTBDRplus(ver 2.0)

Hybridization was performed manually using Twin incubater / shaking water bath at 45 degree as per manufacturer's instruction.

Observation and Result

During this study period out of 800 sputum samples processed for Z-N staining, 168 sample were positive Acid Fast bacilli. *M.tuberculosis* complex was positive in 158 cases out of 168 and 10 samples were detected as Non Tuberculous Mycobacterium. All 158 *M.tuberculosis* isolated were processed for drug sensitivity by PCR and Line Probe Assay.

Table 1. Distribution of drug sensitivity pattern in smear positive cases

Drug Sensitivity pattern	Number of sample
Rifampicin mono-resistant	33
Isoniazid mono-resistant	38
Sensitive to both drugs	125
NTM	10

In this study, all smear positive cases are divided in 2 categories. 43 cases were in category first and 125 in category second. The patient in category first had not taken antitubercle drugs previously, that is they all were new cases. In category 2 all patient had previously received anti-tubercle drugs. Category second patients were further classified as defaulter i.e, those who left treatment in between the course of DOTS therapy.

Table 2. Category wise distribution of patient.

Category	Number of patients		Total no. of patient
	Male	Female	
1	30(24%)	13(30.23%)	43(25.59%)
2	95(76%)	30(69.76%)	125(74.40%)
	125	43	168

Resistance status as shown in table 3 and 4.

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

	Organism	P -value	RIF mono resistant	P- value	ISO mono resistant	P- value	Sensitive to both drugs	p-value
Male	19 (86.36%)	>0.5	19 (86.36%)	>0.5	19 (86.36%)	>0.5	77 (61.6%)	0.077
Female	3 (13.63%)		3 (13.63%)		3 (13.63%)		38 (30.4%)	0.15
Total	22		22		22		115	

Table 4. Age wise distribution of multi drug resistance cases.

Age	AFB GRADING	ORGANISM	RIFAMPICIN	ISONIAZID
15-24	15	15	15	15
25-34	20	20	19	19
35-44	34	34	31	32
45-54	41	41	38	41
55-64	38	38	36	36
65-74	12	12	12	12
75-84	4	4	4	4
85-94	3	3	3	3
Grand Total	167	167	158	162

We also found a higher rate of MDR, RIF and INH mono resistance among defaulter category and sensitivity to both first line drugs was seen highest in new patients category.

Table 5. MDR and Mono resistance pattern in relation to tuberculosis treatment history.

Drug resistant pattern	MDR n(%)	RIF mono resistant n(%)	INH mono resistant n(%)	Sensitive to both drugs n(%)	P- value
New n=49	01(7.69%)	02(15.38%)	01(5.88%)	44(39.40%)	
Failure n=25	02(11.53%)	03(26.07%)	04(29.40%)	14(10.21%)	0.5
Relapse n=33	05(26.52%)	02(15.38%)	03(17.64%)	24(18.97%)	0.0072
Defaulter n=61	9(53.80%)	05(46.15%)	06(47.05%0	43(31.38%)	
Total=168	17	12	14	125	

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

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

Mutation	ORGANISM	RIFAMPICIN	ISONIAZID
531571	8	8	8
S5311	4	4	4
531572	1	1	1
S5311	1	1	1
5315T1	13	12	13
D516V	2	2	2
S531L	6	6	6
5315T3	2	2	2

5531L	1	1	1
L511P	1	1	1
C15T	1	1	1
CIST	2	2	2
D516V 5531L	1	1	1
S315T1	7	7	7
S531L	7	7	7
5531L	3	3	3
D516V	1	1	1
S5311	3	3	3
S531L	4	4	4
Grand Total	68	67	68

DISCUSSION

In this study, the rate of TB infection was found to be more in male.Similarly, high male to female ratio was reported by Singhal et al[11] and Rao[12]. 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 168 more than 50% of patients belong to 26-55 years age group similar findings were reported by Rao[12] WHO also reports that TB mostly affects adults in their most productive years[13].

In present study, MDR TB rate found comparatively higher to previously reported by some authors from India like 4.7% reported by Gupta H et al[14] from Lucknow in 2013. 4.5% reported by Malhotra et al[15] from Jaigurin other studies, such as 14.6% reported by Thakur et al,[16] 2002 whereas it is quite low when compared few et al from Solan H.P in 2015. 21.0% reported by Ahmad et al[17]. from Aligarh in 2017 which indicates rate of MDR-TB vary from region to region.

Simultaneously, rate of Rifampicin and Isoniazid mono-resistance found in this study was 19.6% and 22.6% respectively, while other study of india RIF mono resistance rate of 5.8%, 19.4%, 5.4%, 8.8% frome Aligarh[17], Solan[16], Varanasi[18], and Delhi respectively INH mono resistance from same study 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 more morbidity and mortality. Many drug resistance surveys have shown that mono and poly resistance TB are actually more common than MDR TB[19].

In this study it was also observed that prevalence rate of MDR-TB is quite high in failure, relapse, defaulter cases as compared to new cases. Prevalence rate for failure cases of MDR was 11.53%, for defaulter cases it was 53.80% and 26.52% for relapse cases which is quite high as compared to prevalence rate of MDR in new cases which was 7.69%. These findings support previously reported findings of Thakur et al[16], Malhotra et al[15], Ahmad et al[17], and Talesse et al.

Thakur C et al[16]. in 2015 reported most common mutation is associated with rpoB region mostly S531L mutation we found also S531L mutation as the most common mutation associated with rifampicin resistance. Similar finding were reported by Hirani et al[20], and Sharma et al[21]. Most common mutation associated with isoniazid mono-resistance was S315T. Similar findings were reported by Swaminathan et al[22], and Meeza et al[23].

CONCLUSION

The author found that there is high rate of INH mono resistance which was untreated o rifampicin mono resistance so, interference of MDR based on RIF mono resistance may be an inaccurate strategy to manage patients. All TB patients infected with rifampicin mono resistance strains should be treated using a full MDR TB regimen with isoniazid being added in the regimen until DST result to isoniazid are available. These findings indicate a notable burden of drug resistance requiring routine DST and strengthened diagnostic pathways.

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