DOI: 10.5958/j.2319-5886.3.2.078

International Journal of Medical Research & Health Sciences

www.ijmrhs.com Volume 3 Issue 2 (April - Jun) Coden: IJMRHS Copyright @2014 ISSN: 2319-5886

Received: 12th Feb 2014 Revised: 10th Mar 2014 Accepted: 12th Mar 2014

Research Article

MOLECULAR DETECTION OF RIFAMPICIN AND ISONIAZID RESISTANCE IN CULTURE ISOLATES OF NEWLY DIAGNOSED TB PATIENTS

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ABSTRACT

Introduction: Multidrug-resistant tuberculosis (MDR-TB) is an emerging public health problem in many regions of the world, particularly in developing nations. Accurate and rapid diagnosis is essential in the management of MDR-TB, not only to optimize treatment but also to prevent transmission. **Aims:** To evaluate drug resistance in culture isolates by conventional and molecular methods and detect drug resistance gene in MDR-TB patients. **Material and Method**: 100 newly diagnosed pulmonary tuberculosis (TB) diagnosed patients attending TB Clinic, Gandhi Hospital, Secunderabad were included in the study. Two sputum samples collected from the patients were subjected to sputum microscopy, culture, Drug Susceptibility Testing (DST). Geno Type Mycobacterium Tuberculosis Drug Resistance (MTBDR) *plus* assay was done on the culture isolates to detect Rifampicin and Isoniazid (INH) resistance. **Results:** Out of 100 samples, 48 % smear positivity by Ziehl Neelsen (ZN) method, 51 % culture positivity on LJ medium,11.7% multi drug resistance for Rifampicin and Isoniazid with conventional drug susceptibility – Proportion method,17.6 % drug resistance by molecular method – Geno Type MTBDR plus was observed. Among the 4 Rifampicin (Rif) resistant isolates 2isolates showed mutation (mut) at D516V and in other 2 isolates only wild type (WT) was missing but no mut was seen . In the 1 Isoniazid (INH) resistant isolate WT was missing, but no mutation was seen. Among the 4 Rif +INH resistance all showed mut at S531L for RIF and at S315T1. **Conclusion:** The Genotype MTBDR assay is a rapid and reliable tool for the routine direct detection of MTB strains and of strains resistant to INH and RIF in smear positive, highly infectious patients. The rapid turn around time of the test enables the optimization of the therapy of these patients before confirmatory culture results are available. The test does not require viable organisms and thus reduces the biohazard risk in the laboratory.

Keywords: Mycobacterium Tuberculosis**,** Drug Resistance, Genotype MTBDR assay.

INTRODUCTION

Tuberculosis is the leading cause of mortality in adults due to an infectious agent and accounts for 26 % of all preventable adult deaths globally.[1] At present global incidence of this disease is increasing at the rate of 0.4% per year. It is currently regarded as the seventh most important cause of premature mortality and is going to be one of the first ten leading causes of disease burden even in the year 2020.[2] In India, out of one billion population, each year about two million develop active tuberculosis and up to half million die . Its prevalence and incidence in India is 30.4% and 1.2% respectively.[3]

Multidrug-resistant tuberculosis (MDR-TB) is an emerging public health problem in many regions of

the world, particularly in developing nations. Multi-drug resistant tuberculosis strains are generally considered to be those resistant to at least two drugs,such as INH and Rifampicin. From a microbiological perspective, the resistance is caused by a genetic mutation that makes a drug ineffective against the mutant bacilli. MDR-TB is a man-made phenomenon – poor treatment, poor drugs and poor adherence lead to the development of MDR-TB.[4]The frequency of multi drug resistance varies geographically and acquired resistance is more common than primary resistance.

Accurate and rapid diagnosis is essential in the management of MDR-TB, not only to optimize treatment but also to prevent transmission. Mutations confined to a short 81 bp DNA region in the *rpoB* gene, encoding the ß-subunit of the RNA polymerase, have been found in 95% of Rifampicin-resistant strains. Mutations in this region are an excellent marker for MDR-TB.[4] The successful treatment of tuberculosis depends on timely diagnosis and selection of an adequate treatment strategy. Use of molecular techniques decreases the time necessary for the detection of drug resistance from several weeks to a few days or even less, and a patient's treatment regimen can be adjusted more rapidly to account for any detected drug resistance .[5]

MATERIALS AND METHODS

Sample size: Two early morning sputum samples from 100 cases of clinically suspected newly diagnosed adult pulmonary tuberculosis attending TB clinic, Gandhi Hospital, Secunderabad for over a period of one year were included in the study. 20 cases of clinically non tuberculous etiology were included as controls. The study was approved by Institutional Ethics committee of Gandhi Medical College, Secunderabad

Inclusion criteria: (more than any two of the below to be fulfilled) Fever and cough with expectoration for more than 3 weeks not responding to antibiotics, Gradual weight loss, Loss of appetite, Abnormal findings in chest radiograph

Exclusion criteria: Cases already on anti tuberculosis treatment (ATT) or had been confirmed as having Tuberculosis were excluded.

Collection of sample: Two early morning sputum samples were collected in a sterile leak proof container.

All the samples were subjected to decontamination and concentration **by** Modified Petroff's method [6] Smears were made from the purulent portion of sputum and stained by Ziehl Neelsen method for Microscopy and grading was done as follows (Table 1)

Table 1: Grading of sputum smears

No. Of AFB seen	Result	grading	Fields examined
10 AFB/OIF	Positive	3+	20
1-10AFB/OIF	Positive	2+	50
10-99AFB/OIF	Positive	1+	100
1-9AFB/OIF	Scanty	Record exact number	200
NO. OF AFB in 100 OIF	negative	-----	100

Note: AFB – Acid fast bacilli, OIF – oil immersion field

All the samples were inoculated onto Lowenstein Jensen (LJ) media and incubated at 37°C for a maximum of 8 weeks. In case of any growth of Mycobacteria, date of first appearance of colony was noted and was further incubated for further growth. All the culture positive strains were identified by Para Nitro Benzoic Acid tests and Nitrate reductase test. [6]

Drug Susceptibility testing was done by the molecular method - GenoType®MTBDR plus assay and compared with conventional –Proportion method.

Proportion Method [6]: The proportion method calculates the proportion of resistant bacilli present in the medium with the drug. Two appropriate dilution of the bacilli, 10^{-2} and10^{-4}dilutions (undiluted = 106 to 108 CFU/ml), were inoculated on drug-containing and drug-free media, in order to obtain countable colonies on both media. The ratio of number of colonies observed on the drug -containing media to drug-free medium indicates proportion of resistant bacilli present in the strain. Drug Concentration of Rifampicin added to LJ Media was 40 µg/ml and INH added to LJ media was **0.2** µg/ml .

Incubation and Reading: Inoculated slopes were incubated at 37°C. Growth is read at 28 days.

Growth is recorded Confluent = 3+; More than 100 colonies = 2+; Record actual number of colonies = 1-100 cols.[6]

Interpretation of the test

1. First reading is taken at 28th day after inoculation.
2. Colonies only on the slopes are counted.

3. The average number of colonies obtained from the drug-containing slopes indicate the number of resistant bacilli contained in the inoculum.
4. Dividing the number of colonies in drug containing slopes by that in drug free slopes gives the proportion of resistant bacilli existing in the strain. Below a certain value – the critical proportion – the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages.
5. If, according to the criteria indicated below, the result of the reading made on the 28th day is "resistant", no further reading of the test for that drug is required: the strain is classified as resistant. If the result at the 28th day is "sensitive", a second reading is made on the 42nd day only for the sensitive strain.
6. If growth on the control media is poor even after six weeks (i.e., few or no colonies on the 10-4 bacterial dilution), the test should be repeated.

GenoType® MTBDR plus (Hain Lifescience,)

Methodology

The Geno Type MTBDR plus test is based on the DNA STRIP technology and permits the molecular genetic identification of the Mycobacterium tuberculosis complex and its resistance to Rifampicin and/or Isoniazid from cultivated samples or pulmonary smear-positive clinical specimens. The identification of Rifampicin resistance is enabled by the detection of the most significant mutations of the rpoB gene (coding for the β-sub-unit of the RNA polymerase). For detection of high level Isoniazid resistance, the katG gene (coding for the catalase peroxidase) is examined and for detection of low level Isoniazid resistance, the promoter region of the INHA gene (coding for the NADH enoyl ACP reductase) is examined.

Procedure:

It is divided into three steps-

1. DNA extraction
2. A multiplex amplification with biotinylated primers
3. Reverse hybridization.

Procedure was done and results were interpreted according to the protocol provided by the manufacturer.[7]

RESULTS

Age wise distribution of cases shown in Table 2. The majority of the patients were found to be in the range of 30-39 yrs (34%). Out of 100 patients included in the study, 71 were males and 29 females. Comparison of results of microscopy versus culture on LJ media (Table 3). In this study 44 (48 %) were smear positive by Ziehl Neelsen (ZN method), 55 (51 %) were culture positive on LJ medium. All the isolated strains belonged to Mycobacterium Tuberculosis (MTB) complex. The minimum time taken for growth on LJ media by any strain was 17 days and maximum period taken was 39 days. Maximum number of strains (34) showed visible growth between 22- 28 days. Mean duration of incubation for isolation was 25.66 days.

6 (11.76%) isolates showed drug resistance by convention method (proportion method). Out of 6 drug resistant isolates, 3 isolates showed resistance to only Rif and 3 isolates showed resistance to both drugs.

Out of 51 isolates, 6 isolates (11.7%) were multi drug resistant for Rifampicin and Isoniazid with conventional drug susceptibility – Proportion method, and 9 isolates (17.6%)were drug resistant by molecular method – Geno Type MTBDR plus . The ratio of resistance to both drugs by the two methods is 3:4

Among 4 Rif resistant isolates - 2 showed mut at D516V and in remaining 2 isolates only WT was missing, but no mut seen.1 INH resistant isolate showed WT missing but no mut was seen Among 4 Rif +INH resistant isolates - All showed mut at S531L for Rif, and at S315T1 for INH

Table 2: Age wise distribution of cases

Distribution of age in yrs	No. of cases	percentage
0-9	01	1%
10-19	10	10%
20-29	19	19%
30-39	34	34%
40-49	13	13%
50-59	12	12%
60 and above	11	11%
total	100	100%

Table 3: Comparison of results of microscopy versus culture on LJ media

	Smear +ve	Smear -ve	total
Culture +ve	42	13	55
Culture -ve	02	43	45
total	44	56	100

Table 4: Comparison of drug resistance by conventional dst and genotype mtbdr *plus* method

TEST	Rifampicin Only	Rifampicin + INH	INH only	Total
DST	3	3	-	6
Geno Type	4	4	1	9

Table 5: ANALYSIS OF RIF AND INH RESISTANCE

Sample no	16	28	46	52	62	64	73	83	98
rpoB WT Missing	3,4	8	8	3,4	-	8	8	7	7
rpo B mut	MUT 1	MUT 3	MUT3	MUT 1	-	MUT3	MUT3	NO MUT	NO MUT
Codon analysed	513 - 519	530-533	530-533	513 - 519	-	530-533	530-533	-	-
Mutation	D516V	S531L	S531L	D516V	-	S531L	S531L	-	-
katG WT Missing	-	Missing	Missing	-	Missing	Missing	Missing	-	-
kat G mut	-	-	MUT1	-	-	MUT1	MUT1	-	-
Codon analysed	-	-	315	-	-	315	315	-	-
Mutation	-	-	S315T1	-	-	S315T1	S315T1	-	-
inh A WT	-	-	-	-	-	-	-	-	-
inh A MUT	-	-	-	-	-	-	-	-	-
Codon analysed	-	-	-	-	-	-	-	-	-
Mutation	-	-	-	-	-	-	-	-	-
RESISTANCE	Rif	Rif+INH	Rif+INH	Rif	INH	Rif+INH	Rif+INH	Rif	Rif

Note: WT-wild type; MUT-Mutation; KatG-gene; Rif- Rifampicin; INH- Isoniazid

DISCUSSION

Drug resistance is a threat to TB control programs. It is a major public health problem because treatment is prolonged and complicated, cure rates are well below those for drug- susceptible TB, and patients may remain infectious for months or years, despite receiving the best available therapy. Rapid detection of drug resistance would help not only to optimize treatment of MDR-TB, but also breaking the chains of transmission and identification of any hot spot regions for proper implementation of the TB control programs.

The youngest patient included in this study was 21 years old while the oldest was 80 years old. Maximum number of patients suffering from tuberculosis were in the age group of 30-39 years (34%) followed by 20-29 years (19%). Thus more than half (53%) of patients were in the age group of 20-39 years. Robert etal[7] reported 62% of cases.

In this study out of 100 cases, 71 were males and 29 females. The male to female ratio was 2.4:1 which is in accordance with the study conducted by V.K.Dhingra [8] who reported 2.2:1.

The smear positivity in this study was 42% by standard ZN staining. S Rishi etal [9] reported 54.3% smear positivity while Negi SS etal [10] reported 33.79% smear positivity.

The present study showed 51% culture positivity on LJ media, which correlated with a study conducted by Rishi et al [9] who reported 50.6%. The mean duration of incubation time for Mycobacteria on LJ media was 25.6days while Rishi s etal[9] and Negi S et al [10] reported 28.8 and 24 days as mean duration of isolation respectively.

This study showed cavities in 48% of patients which is in comparison with Dhingra et al [8] who reported 44% on chest X ray.

In this study it was found that 3 isolates (5.88 %) were resistant to Rifampicin alone and 3 isolates (5.88 %) were resistant to both Rifampicin and INH by Proportion method. None of the isolates showed resistance to INH alone. This study correlated with W.C Yam et al [11] who reported 39 to be multi drug resistant among 352 isolates 18 isolates (5.11 %) were resistant to only Rif and 21 isolates (5.96 %) resistant to both the drugs. Meera Sharma et al [12] conducted study on 200 isolates and 14 isolates (7.0%) were resistant to only Rif and 15 isolates (7.5 %) resistant to both the drugs, and 1 isolate (0.5 %) showed resistant to only INH. Naga Suresh et al [13]studied 56 isolates Out of which 5 isolates (8.9 %) were resistant to only Rif and 3 isolates (5.35 %) were resistant to both the drugs.

In this study the 51 isolates were subjected to Geno Type MTBDR plus. 4 isolates (7.8 %) were resistant to Rifampicin, 4 isolates (7.8 %) were resistant to both Rifampicin and INH and 1 isolate (1.96 %) was resistant to INH alone. 4 isolates showed absence of rpoB WT8, codon 531 with rpoB MUT3 i.e mutation at S531L with absent katG WT , codon 315 showing katG MUT 1 mutation at S315T1 . 2 isolates showed absence of rpoB WT 3 ,4 with rpoB MUT 1 i.e mutation at 513 -519 codon mutation at D516V 2 isolates showed absence of rpoB WT7 with no mutation.1 isolate showed absence of only katG WT with no mutation .

This study correlated with Bahram Nasr et al [14]who reported 3 isolates (7.2 %) resistant to Rifampicin and 6.8 % to both the drugs , with absence of rpoBWT8 with rpoB MUT3 i.e mutation at S531L and absent katG WT showing katG MUT 1 mutation at S315T1 and absence of rpoB WT 3 ,4 with rpoB MUT 1, mutation at 513 -519 codon i.e mutation at D516V was seen. Doris Hillemann et al [15]reported 9.2 % of strains resistant to Rifampicin and 8.1 % isolates showed resistance to both the drugs. Mutations were seen in 531 codon for rpoB 315 for katG, 13.6 % and in 526, 516 codon.5 isolates showed absence of wild types with no mutation. Paolo Miotto et al [16] reported in 3.8 % of isolates showed D516V substitution in rpoB and 4.4 % in S531L region.

In this study with Proportion method drug resistance was seen in 6 isolates and with rapid molecular method Geno Type MTBDR plus 9 isolates were drug resistant. As compared to the conventional method which showed 3 isolates to be resistant to both the drugs, the Geno Type MTBDR plus assay showed 4 isolates to be resistant to both the drugs. 3 isolates were resistant only to Rifampicin by conventional method and 4 isolates showed resistance only to Rifampicin by the GenoType MTBDR plus assay, the ratio being 3:4. Our study correlated with Doris Hillemann et al [15]who reported 3.2: 4 ratio in comparison of both methods. Guessan Kouassi et al [17] showed 1: 6 ratios. Girts Skenders et al 18 reported 1:7 ratio of both methods. In addition, one isolate showed resistance to INH alone by GenoType MTBDR plus method which was not shown by Proportion method.

CONCLUSION

The results of the present study have shown that the MTBDR plus assay is easy to perform and has the capability for the rapid detection of Rifampicin - and INH-resistant M. tuberculosis. MTBDR plus assay has been proven to be suitable for application for culture isolates .MTBDR assay can identify the most frequent mutations involved in resistance to RIF and INH and can reveal the presence of additional mutations by negative hybridization results with the wild-type probes. MTBDR assay identified 100% of the phenotypically resistant strains for Rifampicin and Isoniazid resistance.

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