International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

The Efficacy of Genotype® MTBDR*plus* Assay in Rapid Detection of Rifampicin and Isoniazid Resistance in *Mycobacterium tuberculosis* Complex Isolates

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Abstract: ***Aims: To evaluate the efficacy of GenoType® MTBDRplus assay in rapid detection of Tuberculosis cases resistant to Isoniazid and/or Rifampicin and to detect the drug resistant genes. Materials and Methods: Early morning sputum samples from 100 clinically suspected TB patients were collected from south Gujarat region and subjected to sputum microscopy, culture, Drug Susceptibility Testing (DST). GenoType Mycobacterium Tuberculosis Drug Resistance (MTBDR) plus assay was done on the culture isolates to detect Rifampicin and/or Isoniazid resistance. The efficacy of the Genotype MTBDRplus assay was compared with that of the phenotypic drug susceptibility test-1%Proportion method. Results: Out of 100 patients, 27 were smear positive by Ziehl-Neelsen method and 30 were culture positive on Lowenstein Jensen medium. Out of 30 isolates, 7 isolates (23.3%) showed drug resistance by conventional DST method while 8 isolates (26.67%) showed drug resistance by the Genotype MTBDRplus. Out of 8 isolates, 3 isolates (37.5%) showed drug resistance to only Isoniazid, 1 isolate (12.5%) showed drug resistance to only Rifampicin and 4 isolates (50%) were multi-drug resistant for Rifampicin and Isoniazid with molecular method-Genotype MTBDRplus. Conclusion: The MTBDRplus assay allows the rapid detection of drug resistance patterns with significantly lesser turnaround time as compared to conventional DST method.***

Keywords: *Mycobacterium tuberculosis*, Drug resistance, Multi-drug resistant, Proportion method, Genotype MTBDRplus assay

1. Introduction

Tuberculosis (TB) is an ancient disease that has affected mankind for more than 4,000 years[1]. TB, one of the world's deadliest communicable diseases, remains a leading cause of morbidity and mortality in developing countries, including India. Though India is the second-most populous country in the world, one fourth of the global incident TB cases occur in India annually[2]. India accounts for more than 25% of the world's incident cases of tuberculosis[3]. India is a high tuberculosis (TB) burdened country with increasing prevalence of multidrug resistant tuberculosis (MDR TB). In 2012 there were an estimated 170,000 deaths from MDR TB, and 450,000 new cases of MDR-TB.[4]

Multidrug-resistant tuberculosis (MDR-TB) is a disease caused by *Mycobacterium tuberculosis* strains with resistance to, at least, two first line drugs Isoniazid and Rifampicin. It is a major public health problem that threatens progress made in TB care and control worldwide. The development of drug resistance in *M. tuberculosis* isolates is due to the random genetic mutations in particular genes that confer resistance[5]. Based on this information, the molecular assays have been established for rapid detection of drug resistance in clinical *M. tuberculosis* isolates within a day[6].

The mutations mainly found in Rifampicin (RMP)-resistant *M. tuberculosis* isolates are located in an 81-bp "core region" of the *rpoB* gene [Fig. 2][7, 8]. However, the mutations causing Isoniazid (INH) resistance are located in several genes and regions[9]. About 50 to 95% of INH-resistant strains have been found to contain mutations in codon 315 of the *katG* gene[10,11,12]. While 20 to 35% contain mutations in the *inhA* regulatory region[11,12,13]. Mutations in these regions are an outstanding marker for detection of MDR-TB cases. Spread of Multi drug-resistant TB is a man-made problem mainly related to poor case management and lack of quality drugs[14]. Timely diagnosis and appropriate treatment of infectious cases are the only key elements in reducing the spread of TB.

GenoType MTBDR*plus* assay is a molecular technique which allows the direct identification of *M. tuberculosis* complex and its resistance to Rifampicin (RMP) and/or Isoniazid (INH) from culture isolates or smear-positive pulmonary clinical specimens within one working day, thus saving several weeks of time required for culture and DST[15].

The main aim to perform this study was to evaluate the efficacy of GenoType® MTBDR*plus* assay in rapid detection of TB cases resistant to INH and/or RIF and to detect the drug resistant genes.

2. Materials and Methods

Sample Size

Early morning sputum samples from 100 clinically suspected TB patients were collected from different hospitals of south Gujarat region, India, a period of one year from June 2013-May 2014. This study was done after approval from the local research ethics committee. All Mycobacterial investigations were carried out at the Microcare Tuberculosis Laboratory, Surat. The laboratory is accredited for carrying out culture and Drug Susceptibility Testing (DST) by the Central TB Division, Ministry of Health and Family Welfare, Govt. of India.

Sample Collection and Processing:

Early morning sputum samples were collected in a sterile, leak-proof container. All the specimens were handled in class II bio safety cabinet in a bio-safety level (BSL) – 3 laboratory and were decontaminated by Modified Petroff's Method[16].
All the samples were subjected to smear examination for detection of acid fast bacilli (AFB) and culturing. Smears were made from the mucopurulent portion of sputum and stained by the conventional Ziehl Neelsen method. The smears were graded according to the number of bacilli seen on the slide, as per recommendations of the World Health Organization (WHO). For culturing of the specimen, two McCartney Bottles of Lowenstein – Jensen medium were inoculated with each sample and incubated at 37ºC until growth of mycobacterium was observed or were discarded as negative after 8 weeks. All the culture isolates were identified as *Mycobacterium tuberculosis* by their slow growth rate, colony morphology, inability to grow on L-J media containing p-nitro benzoic acid (500 mg/ml), niacin positive and catalase negative tests[16].
Drug Susceptibility Testing was done by the Genotype MTBDRplus assay and the efficacy of this molecular method was compared to that of the phenotypic drug susceptibility test – 1% proportion method.

GenoType® MTBDR*plus* assay (Hain Lifescience, Nehren, Germany)

The GenoType® MTBDR*plus*, a commercially available DNA Strip, assay was performed as recommended by the manufacturer (Hain Lifescience, Nehren, Germany).

This DNA Strip based technology allows the molecular identification of the Mycobacterium tuberculosis complex and its mutations that confer resistance to Rifampicin and/or Isoniazid from pulmonary smear-positive clinical specimens or cultivated samples.

The GenoType® MTBDR*plus* assay includes the following three steps -
1. DNA extraction
2. Multiplex polymerase chain reaction (PCR) amplification
3. Reverse hybridization.

These steps were performed in three separate rooms with restricted access and unidirectional workflow. Mycobacterial DNA extraction was done in BSL-3 laboratory according to manufacturer's instructions (Hain Lifescience, Nehren, Germany). 5 µl of the extracted supernatant DNA was used for PCR while the remainder was stored at -20ºC.

For amplification, master mixture was prepared. This master mixture consisted of 35 µl primer nucleotide mixture (provided with kit), 5 µl of 10XPCR buffer with 15 mM MgCl2, 2 µl of 25 mM MgCl2, 0.2 µl (1 U) of HotStarTaq DNA polymerase (Hain Lifescience, Nehren, Germany), 3 µl nuclease free molecular grade water and 5 µl of DNA supernatant in a final volume of 50 µl. The amplification protocol consisted of 15 min of denaturation at 95°C, followed by 10 cycles comprising 30 s at 95°C and 120 s at 58°C; an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C; and a final extension at 70°C for 8 min.

Hybridization was performed with the automatic machine (twincubator). After hybridization and final washing, strips were removed, air dried, fixed on paper and results were interpreted according to the protocol given by the manufacturer (Hain Lifescience, Nehren, Germany).

The GenoType® MTBDRplus strip contains 17 probes, including amplification and hybridization controls for the verification of the test procedures. For the detection of Rifampicin (RMP) resistance, eight *rpoB* wild-type probes (probes WT1 to WT8) encompass the region of the rpoB gene encoding amino acids 509-533. Four probes (probes *rpoB* MUT D516V, *rpoB* MUT H526Y, *rpoB* MUT H526D and rpoB MUT S531L) specifically target the most common mutations conferring resistance to Rifampicin. For the detection of Isoniazid (INH) resistance, one probe cover the wild-type S315 region of *katG*, while two others (probes *katG* MUTT1 and MUTT2) are designed to assess the AGC-to-ACC (S315T) and the AGC-to-ACA (S315T) mutations. Moreover, the promoter region of the inhA gene is included on the new strip and encompasses the regions from positions -15 to -16 for the *inhA* WT1 probe and positions -8 for the *inhA* WT2 probe. Four mutations (-15C/T, -16A/G, -8T/C and -8T/A) can be targeted with the *inhA* MUT1, MUT2, MUT3A and MUT3B probes. Either the missing of wild-type band(s) or the presence of mutant band(s) indicates a resistant strain [Fig.1][17].

Conventional Drug Susceptibility Testing

The Conventional Drug Susceptibility Testing -1% proportion method was performed on Lowenstein-Jensen solid medium according to the standard operating procedure of RNTCP[18]. Since the proportion method is widely used across laboratories in India under RNTCP as a standardized method for DST, this method was used as a reference standard method in this study.

The Proportion method enables precise estimation of the proportion of resistant mutants present in the Lowenstein-Jensen medium with the drug. The first line drugs like rifampicin, isoniazid, streptomycin and ethambultol were tested with concentrations of 40 µg /ml, 0.2µg/ml, 4µg/ml and 2µg/ml respectively. Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – rifampicin, isoniazid, streptomycin and ethambultol – was classified as resistant to that drug[18].

3. Results

Demographic data has been shown in Table 1 and 2. The majority of the patients were found to be in the age group of 30-39 years (31%) [Table1]. 100 patients were included in the study. Out of those, 61 were males and 39 were females [Table 2]. Out of 100 patients, 27 were smear positive by Ziehl-Neelsen method (27%), 30 were culture positive on Lowenstein Jensen medium (30%). All positive cultures belonged to Mycobacterium Tuberculosis (MTB) complex. The minimum time taken for the growth on Lowenstein Jensen medium was 20 days and the maximum was 41 days.

Out of 30 isolates, 7 isolates (23.3%) showed drug resistance by conventional DST method. Out of 7 drug resistance isolates, 3 isolates (42.85%) showed drug resistance to only Isoniazid, 1 isolate (14.28%) showed resistance to only Rifampicin and 3 isolates (42.85%) were multi-drug resistant for Rifampicin and Isoniazid with conventional drug susceptibility-Proportion method [Table 3].

Out of 30 isolates, 8 isolates (26.67%) showed drug resistance by molecular method-Genotype MTBDR*plus*. Out of 8 drug resistance isolates, 3 isolates (37.5%) showed drug resistance to only Isoniazid, 1 isolate (12.5%) showed drug resistance to only Rifampicin and 4 isolates (50%) were multi-drug resistant for Rifampicin and Isoniazid with molecular method-Genotype MTBDR*plus* [Table 4].

4. Discussion

TB, one of the world's deadliest communicable diseases, remains a leading cause of morbidity and mortality in developing countries, including India. It is the first infectious disease declared by the World Health Organization (WHO) as a global health emergency[19]. The death rate in MDR cases is high (50-60%) and is often associated with a short span of disease (4-16 weeks) [20]. In recent years, more emphasis has been given on rapid diagnosis and prompt treatment of MDR-TB. In this context, we evaluated the MTBDR*plus* assay in an area with high TB incidence. The MTBDR*plus* assay has been proven to be suitable for application for both with culture isolates and smear positive specimens. It allows the rapid detection of Rifampicin and/or Isoniazid resistant *Mycobacterium tuberculosis*.
The youngest patient included in this study was 9 years old while the oldest was 65 years old. Maximum number of patients were found to be in the age group of 30-39 years (31%) followed by 20-29 years (18%). In this study, out of 100 cases 61 were males and 39 were females. Men are more commonly affected than women. The case notifications in most countries are higher in males than in females[21].

Out of 100 patients, 27 were smear positive by Ziehl-Neelsen method (27%), 30 were culture positive on Lowenstein Jensen medium (30%). In this study, 30 isolates were subjected to Genotype MTBDR*plus* assay, 1 isolate (3.33%) was resistant to Rifampicin, 4 isolates (13.33%) were resistant to both Rifampicin and Isoniazid (MDR) and 3 isolates (10%) were resistant to Isoniazid. The detection of mutations in the *rpoB* gene is a useful strategy for diagnosis of drug resistance to Rifampicin in *M. tuberculosis* complex since the mutations in the hot spot region are predominant (average,>95%) [Fig. 2][22]. Additional analysis of codon 315 of *katG* facilitates the detection of the most-frequent mutations associated with Isoniazid resistance[5].
In this study, 3 isolates (Sample No. 15, 87 and 90) showed absence of rpoB WT 8 with rpoB MUT 3, mutation at 530-533 Codon, i.e., mutation at S531L and absence of katG WT with katG MUT 1, mutation at 315 Codon, i.e., mutation at S315T1. 3 isolates (Sample No. 38, 55 and 57) showed absence of katG WT with katG MUT 1, mutation at 315 Codon, i.e., mutation at S315T1. 1 isolate (Sample No. 52) showed absence of rpoB WT 3, 4 with rpoB MUT 1, mutation at 513-519 Codon, i.e., mutation at D516V and absence of katG WT with katG MUT 1, mutation at 315 Codon, i.e., mutation at S315T1. 1 isolate (Sample No. 36) showed absence of rpoB WT 2 with no mutation [Table 4]. The results of the present study have shown that the Genotype MTBDR*plus* assay is easy to perform and has capability for the rapid and specific detection of the most frequent mutations leading to Rifampicin and Isoniazid resistance in clinical *Mycobacterium tuberculosis* isolates.

In this study, the drug resistance was seen in 7 and 8 isolates with the proportion method and the Genotype MTBDR*plus* assay respectively. As compared to the conventional Proportional method which showed 3 isolates to be resistant to both drugs, the Genotype MTBDR*plus* assay showed 4 isolates to be resistant to both the drugs. In this study Conventional DST-Proportion method was used as the reference standard, and we observed that the MTBDR*plus* test results had a good concordance with the conventional DST with an additional advantage of a shorter turnaround time. Based on our results, the Genotype MTBDR*plus* assay revealed a high sensitivity and specificity for detection of Rifampicin and Isoniazid compared to the conventional DST.
Some authors discussed a main limitation of the MTBDR test system, which is the low sensitivity for the detection of INH resistance[23, 24]. This was due to the fact that the test targets only the *katG* S315T mutation. By using the MTBDR*plus* assay, this problem is partly solved by the addition of a second target, the *inhA* gene, for the detection of INH resistance. Through our study, we could show that rate of detection of Isoniazid resistance was excellent.

The MTBDR*plus* assay is a rapid technique as it can be applied directly to smear-positive specimens. With a turnaround time of approximately 6 hours, these techniques save several weeks of time, this is required for primary isolation and conventional DST.

5. Conclusion

From the results of the present study, it is concluded that the Genotype MTBDR*plus* assay is a reliable, an efficient and a valuable technique for the detection of the mutations leading to Isoniazid and Rifampicin resistance in clinical *Mycobacterium tuberculosis* isolates. The MTBDR*plus* assay allows the rapid detection of drug resistance patterns with significantly lesser turnaround time saving several weeks of time required for culture as compared to conventional DST method and can easily be implemented in routine work flow. In addition, the MTBDR*plus* assay does not require viable organisms and thus reduces the biohazard risk in the laboratory.

6. Acknowledgments

We are thankful to Microcare Laboratory for providing infrastructure and lab-space and Hain Lifescience, Germany, for providing the Genotype MTBDR *plus* assay.

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Table 1: Age-wise distribution of cases

Age Group (Years)	No. Of cases	Percentage (%)
0-9	1	1
10-19	13	13
20-29	18	18
30-39	31	31
40-49	16	16
50-59	11	11
60 and above	10	10
Total	100	100

Table 2: Gender-wise distribution of cases

Sex	**No of patients**
Male	61
Female	39
Total	100

Table 3: Genotype MTBDR*plus* test results in comparison with Conventional DST-Proportion method

Test	Rifampicin only	INH only	Rifampicin + INH	Total
MTBDR *plus*	1	3	4	8
Conventional DST	1	3	3	7

Table 4: MTBDR*plus* test results for the analyses of Rifampicin and Isoniazid resistance

Sample No.	15	36	38	52	55	57	87	90
rpoB WT Missing	8	2	-	3,4	-	-	8	8
rpoB mut	MUT-3	No MUT	-	MUT-1	-	-	MUT-3	MUT-3
Codon analysed	530-533	-	-	513-519	-	-	530-533	530-533
Mutation	S531L	-	-	D516V	-	-	S531L	S531L
katG WT Missing	Missing	-	Missing	Missing	Missing	Missing	Missing	Missing
katG mut	MUT-1	-	MUT-1	MUT-1	MUT-1	MUT-1	MUT-1	MUT-1
Codon analysed	315	-	315	315	315	315	315	315
Mutation	S315T1	-	S315T1	S315T1	S315T1	S315T1	S315T1	S315T1
inhA WT	-	-	-	-	-	-	-	-
inhA mut	-	-	-	-	-	-	-	-
Codon analysed	-	-	-	-	-	-	-	-
Mutation	-	-	-	-	-	-	-	-
RESISTANCE	Rif+INH	Rif	INH	Rif+INH	INH	INH	Rif+INH	Rif+INH

NOTE: WT-wild type; MUT-Mutation; rpoB, katG, inhA-Genes; Rif-Rifampicin; INH-Isoniazid

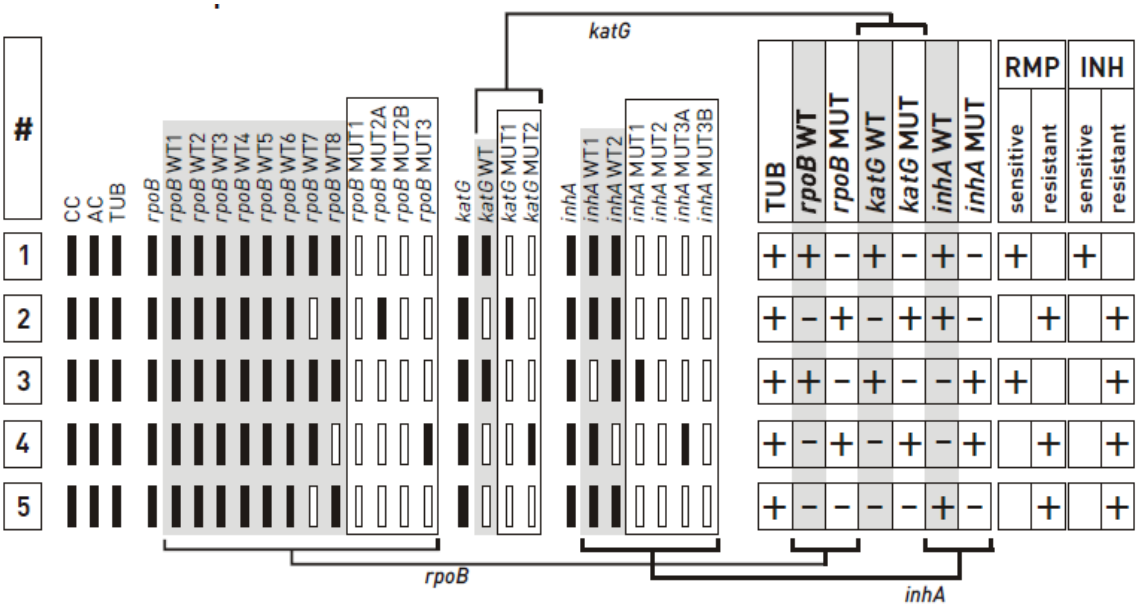

Figure 1: Examples for banding patterns and their evaluation with respect to RMP and/or INH resistance

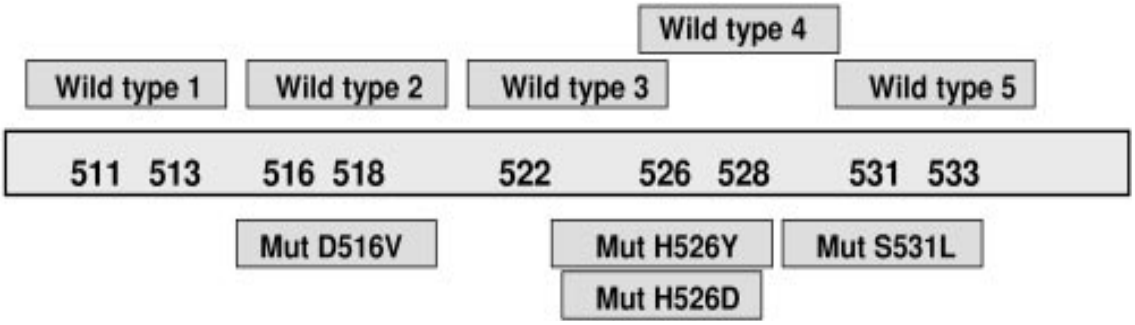

Figure 2: Locations of probes within the 81-bp hot spot cluster of the *rpoB* gene.

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