Molecular Diagnosis of Rifampicin and Isoniazid Resistant Tuberculosis Using GenoType MTBDR*plus* Line Probe Assay from Sputum Samples

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Abstract: **The main objective to perform this study was to evaluate the performance of molecular technique GenoType MTBDR*plus* in rapid diagnosis of drug resistant TB. Drug Susceptibility Testing (DST) was done by the Genotype MTBDR*plus* assay for rapid diagnosis of Rifampicin and/or Isoniazid resistant Tuberculosis and its performance was compared to that of the conventional DST–1% Proportion method. In our study, from 101 TB positive isolates, 37 and 38 isolates were multi drug resistant by Proportion method and MTBDR*plus* assay, respectively. As compared to Proportion method which showed 8 isolates to be resistant to Rifampicin drug, the MTBDR*plus* assay showed only 4 isolates to be resistant to Rifampicin. Sensitivity of the MTBDRplus assay was found to be 50% and 100% for RIF and INH respectively whereas specificity was found to be 100% for both RIF and INH. GenoType MTBDR*plus* is highly specific for molecular diagnosis of MDR-TB with significantly lesser turnaround time, as compared to conventional DST method.**

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

I. INTRODUCTION

Tuberculosis (TB) is one of the world's deadliest communicable diseases caused by the members of *Mycobacterium tuberculosis* complex (MTBC). The disease caused by *Mycobacterium tuberculosis* resistant to two primary anti-tubercular drugs, rifampicin and isoniazid, is known as multi-drug-resistant tuberculosis (MDR-TB). The burden of TB particularly with multi-drug-resistance is increasing worldwide and has become a major public health concern [1, 2]. In 2013, 9 million people around the world fell sick to TB with around 1.5 million TB-related deaths worldwide. Globally, 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB in 2013 (Global TB Report, 2014) [3]. Conventional method is, however, time consuming and often leads to delay in diagnosis of infectious TB cases. The slow diagnosis of drug resistant TB can be a major contributor for the transmission of MDR-TB. Hence, effective control of drug resistant TB relies on rapid diagnostic assays. In 2008, the World Health Organization endorsed the use of GenoType MTBDR*plus* assay, a molecular based line probe assay, which allows simultaneous detection of MTBC and MDR-TB from culture isolates or smear positive pulmonary samples within a day and saves several weeks of time required for culture and Drug Susceptibility Testing (DST) [4]. Resistance to Rifampicin is achieved by detecting most common mutations in the *rpo* gene while resistance to Isoniazid is detected by presence of the most common mutations in the *katG* (confers high- level resistance to Isoniazid) and *inhA* (confers low- level resistance to Isoniazid) genes [5]. Mutations in these genes are an outstanding marker for MDR-TB detection. The main objective to perform this study was to evaluate the performance of molecular technique GenoType MTBDR*plus* in rapid diagnosis of drug resistant TB.

II. MATERIALS AND METHODS

The study was carried out at the Microcare Tuberculosis Laboratory, Surat, which is accredited for carrying out culture and DST by the Central TB Division, Ministry of Health and Family Welfare, Govt. of India. Sputum samples from new pulmonary TB patients were collected and processed for microscopy, culturing and DST.

Microscopy and culture

All sputum samples were collected in a sterile, leak-proof container and were smeared, air-dried, fixed and stained by the conventional Ziehl Neelsen (ZN) method. The smears were graded as per the recommendations of WHO.

All samples were decontaminated using N-Acetyl L-cysteine/Sodium Hydroxide (NALC/NaOH) method [6]. 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 [7].

Drug Susceptibility Testing

DST was done by the Genotype MTBDR*plus* assay and the performance of this molecular method was compared to that of the conventional drug susceptibility test–1% Proportion method.

Conventional Drug Susceptibility Testing

The Conventional Drug Susceptibility Testing–1% proportion method was performed on LJ solid medium according to the standard operating procedure of RNTCP. Drug concentrations tested were 40 µg/ml and 0.2µg/ml for Rifampicin and Isoniazid respectively [8].

GenoType MTBDRplus assay

The GenoType MTBDR*plus* assay was performed as recommended by the manufacturer (Hain Lifescience, Nehren, Germany).

The GenoType MTBDR*plus* assay is based on the DNA Strip technology. The whole procedure is divided into three steps:-

1. DNA extraction
2. Multiplex polymerase chain reaction (PCR) amplification with biotinylated primers
3. Reverse hybridization

The GenoType MTBDRplus strip contains 17 probes, including amplification and hybridization controls for the verification of the test procedures and 10 mutation probes. For the detection of Rifampicin 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 resistance, one probe cover the wild-type S315 region of *katG*, while two others (probes *katG* MUT1 and MUT2) 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 position -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 (Figure 1) [9].

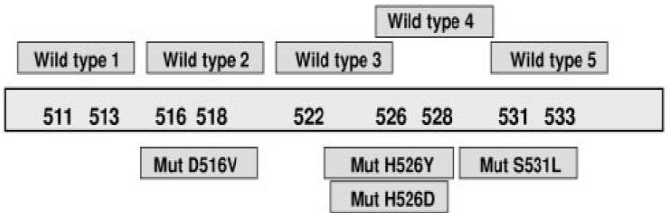

Figure 1: Location of probes within the 81 bp hot spot cluster of the *rpoB* gene

III. RESULTS

This study was conducted from August 2013 to November 2014. Total 234 samples were collected from TB suspected patients of South Gujarat region, out of which 101 patients were found to be TB positive from which 70 were males (69%) and the rest 31 were females (31%).

Out of 234 samples, 94 were smear positive by Ziehl Neelsen method (40.17%) and 140 were smear negative. In the case of culturing, out of the 234 samples, 101 were culture positive on Lowenstein-Jensen medium (43.16%) and 133 were culture negative (Table: 1). In this study, 101 samples were processed for both conventional and genotypic drug susceptibility testing.

Table 1: Results of microscopy versus culture on LJ media

Comparison of results of microscopy versus culture on LJ media			
	Smear +ve	Smear -ve	Total
Culture +ve	94	7	101
Culture -ve	0	133	133
Total	94	140	234

Table 2: Susceptibility Test results for 101 *M. tuberculosis* Strains

DST results	Proportion method	MTBDRplus assay
Susceptible to RIF and INH	47	50
RIF-Monoresistant	8	4
INH-Monoresistant	9	9
MDR	37	38
Total	101	101

In our study, from 101 TB positive isolates, 37 and 38 isolates were multi drug resistant by Proportion method

and MTBDR*plus* assay respectively. As compared to Proportion method, which showed 8 isolates to be resistant to Rifampicin drug, the MTBDR*plus* assay showed only 4 isolates to be resistant to Rifampicin. Thus, the result showed low sensitivity for Rifampicin drug, as not all mutations leading to resistance were detected on the test strip (Table 2). As compared to Proportion method, the sensitivity of the MTBDRplus assay was found to be 50% and 100% for RIF and INH respectively whereas specificity was found to be 100% for both RIF and INH.

Among the resistant isolates studied, 38 strains were reported to be MDR by Genotype MTBDR*plus* assay. Mutation pattern of these isolates was found to be varied with altogether 11 different patterns of mutation. Most prominent mutation of MDR isolates was observed in the 531 gene region of *rpoB* gene, that is, S531L mutation (26/38; 68.42%) for RIF resistance and 315 region of *katG* gene for INH resistance (34/38; 89.47%) (Table: 3).

Table: 3 Multiple gene mutations in MDR-TB isolates using Genotype MTBDRplus Assay

Multiple gene mutations in MDR-TB isolates	No. of isolates (n=38)
rpoB MUT 3, *rpoB* WT 8, *katG* MUT 1, *katG* WT	20
rpoB MUT 1, *rpoB* WT 3, *rpoB* WT 4, *katG* MUT1, *katG* WT	4
rpoB WT 8, *katG* MUT 1, *katG* WT	2
rpoB MUT 3, *rpoB* WT 8, *inhA* MUT 1, *inhA* WT 1	2
rpoB WT 8, *katG* WT	1
rpoB MUT 3, *rpoB* WT 8, *katG* WT	4
rpoB WT 2, *rpoB* WT 3, *rpoB* WT 4, *katG* MUT 1, *katG* WT	1
rpoB WT 3, *rpoB* WT 4, *katG* MUT 1, *katG* WT	1
rpoB WT 3, *rpoB* WT 4, *katG* WT, *inhA* MUT 3A, *inhA* WT 2	1
rpoB WT 3, rpoB WT 4, *katG* WT	1
rpoB WT 8, *inhA* MUT 1, *inhA* WT 1	1

Table: 4 Multiple mutations observed using Genotype MTBDR*plus* assay

LPA probes	Mutation sites	Mutation detected	No. of isolates
rpoB			**RIF-Monoresistant isolates (4)**
WT 7, MUT 2A	526-529	H526Y	4
katG			**INH-Monoresistant isolates (9)**
WT, MUT 1	315	S315T1	8
inhA			
WT1, MUT 1	-15/-16	C15T	1

IV. DISCUSSION

The present study shows that men are more commonly affected than women. It was also found that the case notifications in most countries are higher in males than in females [10]. Our findings were similar to that of Vanisree *et al.* (2014).

The present study shows that Genotype MTBDR*plus* assay has excellent specificity (100%) for detection of RIF and INH-resistant TB and also has excellent sensitivity for detection of INH resistance (100%) but has lesser accuracy for detection of RIF resistance (50%). On the basis of these results, it can be inferred that although Genotype MTBDR*plus* assay has advantage of rapid turnaround time, conventional DST should always be carried out before arriving at conclusive test results.

In our study, discordance of sensitivity of Genotype MTBDR*plus* with conventional phenotypic DST-1% Proportion method was reported. Sensitivity for detection RIF resistance in this study was lower than reported from various meta-analyses [11, 12, 13]. Similarly, sensitivity of the assay for INH resistance in this study was higher than reported in Caribbean (35– 73%) [14], Germany (88.4%) [15] and South Africa 62.1% [13].

In our study, among various drug resistant isolates observed using GenoType MTBDR*plus* assay, majority of them had multiple mutations (Table 3). All RIF resistant

isolates were reported to have their mutations in 81-bp core rifampicin resistance determining region (RRDR) of *rpoB* codons 510 to 533 (Figure 1). This is in agreement with a study done in Belgium [16] and Nepal [17] where over 96% of RIF resistant *M. tuberculosis* strains were reported to have mutations in RRDR of *rpoB* region.

In our study of INH Monoresistant strains, 88.88% of strains have S315T mutation in *katG* region. This is similar to the result found in Thailand where 90% of all Isoniazid resistance isolates had mutation in the *katG* gene [18]. Only 11.11% of the INH Monoresistant strains were observed to have mutation in the *inhA* region (Table 4).
High level and low level of INH resistance were shown to be associated with codon315 of *katG* gene (50–90%) and regulatory region of *inhA* gene (20–35%), respectively, by various studies [19, 20] which corroborates with our study for *katG* mutation but not for *inhA* mutation. Limitations of MTBDRplus assay include the requirement of appropriate infrastructure and skilled laboratory personnel.

V. CONCLUSION

GenoType MTBDR*plus* is a reliable and efficient molecular technique for rapid detection of mutations leading to Isoniazid and Rifampicin resistance. This method is also highly specific for diagnosis of MDR-TB with significantly lesser turnaround time, as compared to conventional DST method.

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