International Journal of Medical Research Professionals

Original Article

Early Detection of Multidrug Resistant Tuberculosis Using Genotype MTBDR*plus* Line Probe Assay in a Tertiary Care Centre, Assam.

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Article History
Received: 26 Feb 2016
Revised: 28 Feb 2016
Accepted: 29 Feb 2016

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ABSTRACT

Introduction: Rapid detection of drug resistance is of particular importance in Multi Drug Resistant tuberculosis (MDR-TB), as conventional methods for mycobacteriological culture and drug susceptibility testing are slow and cumbersome. World Health Organization recommended a new policy of using line probe assays for rapid screening of patients at risk of MDR-TB in 2008. Our study aimed at detecting drug resistant tuberculosis in clinical specimens using GenoType MTBDR*plus* line probe assay (Hain Lifesciences, Germany).

Methods: The study was conducted for a one year period and a total number of 84 pulmonary specimens (viz. sputum, broncho-alveolar lavage (BAL) and pleural fluid) were processed in the Mycobacteriology laboratory for direct microscopy and line probe assay test.

Results: Out of the 84 clinical specimens, 21 showed acid fast bacilli on Ziehl Neelsen staining smear microscopy and also gave valid result for *M. tuberculosis complex* (18 smear positive sputum specimens and 3 BAL) in MTBDR*plus* assay. Out of them, 16 were found susceptible to both Rifampicin (Rif) and Isoniazid (INH), 5 were resistant to both Rif and INH; without any mono-resistance pattern. All the five MDR-TB specimens were sputum and showed mutation band in both *rpo*B and *kat*G; none in *inh*A.

Discussion: The GenoType MTBDR*plus* line probe assay was found to be very useful for rapid diagnosis of TB and drug resistance in both smear-positive and smear-negative specimens. This test also detects monoresistance to Isoniazid and Rifampicin which will aid clinicians to manage cases with MDR-TB.

KEYWORDS: Line probe assay, Multi drug resistant tuberculosis, Mutation.

INTRODUCTION

Drug resistant tuberculosis has emerged as global public health problem. Early diagnosis of tuberculosis (TB) and *Mycobacterium tuberculosis* drug resistance are critical to establish timely initiation of therapy and to avoid therapeutic failure. World Health Organization (WHO) estimates 5% of TB cases to have had multidrug-resistant TB (MDR-TB) globally in 2014. Drug resistant surveillance data show that an estimated 480000 people developed MDR-TB in 2014 and 190000 people died of MDR-TB. India recorded 2.2% of new TB cases with MDR-TB and 15% in retreatment cases in 2014.[1]

Rapid detection of drug resistant TB is of particular importance in MDRTB, which may lead to devastating consequences like the emergence of extensively drug resistant TB (XDR-TB), potential transmission, and rapid mortality of MDR- TB and XDR-TB patient. Conventional methods for mycobacteriological culture and drug susceptibility testing (DST) are slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of *Mycobacterium tuberculosis* complex, and *in vitro* testing of susceptibility to anti-tubercular drugs. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Liquid media-based tests, such as the BACTEC®, and MGIT® systems, are more rapid, but also more costly and require sophisticated laboratories and trained personnel.[2] These limitation led to adoption of novel diagnostic tool like molecular line probe assays (LPA) which permits rapid diagnosis of TB, Isoniazid and Rifampicin resistance.

WHO recommended a new policy of using line probe assays for rapid screening of patients at risk of MDR TB

in 2008 which states the use of commercial line probe assays rather than in-house assays to ensure reliability and reproducibility of results.[3] The GenoType MTBDR*plus* (MTBDR-Plus) (Hain Lifesciences GmBH, Nehren, Germany) is one such commercially available line probe assay based on multiplex PCR combined with reverse hybridization on nitrocellulose strips, targeting common mutations. This assay identifies Rifampicin (RIF) and Isoniazid (INH) resistance by detecting the most common mutations of the *rpoB* gene and the *katG* and *inhA* genes, respectively, and can be used on direct specimens as well as culture isolates.[4]

The aim of our study was to detect drug resistant MTB in clinical specimens using GenoType MTBDR*plus* line probe assay.

MATERIALS AND METHODS

The study was carried out in a tertiary care centre in Assam, India during a period of one year from January, 2015 to December, 2015. A total number of 84 pulmonary specimens viz. sputum, broncho-alveolar lavage (BAL) and pleural fluid received in the Mycobacteriology laboratory which is accredited for performing LPA test were included in the study.

Specimen Collection and Processing

All the specimens were collected in sterile 60 mL screw capped container; handled and processed in class II biosafety cabinet in a bio-safety level (BSL)-3 laboratory. Specimens were digested and decontaminated using N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method; screened for acid fast bacilli using Ziehl-Neelsen (Z N) smear microscopy followed by GenoType MTBDR*plus* line probe assay.

Line Probe Assay

The GenoType MTBDR*plus* VER 2.0 line probe assay was performed according to the manufacturer's (Hain Lifesciences, Nehren, Germany) instructions.[4] Three steps for LPA test included, deoxyribonucleic acid (DNA) extraction, multiplex polymerase chain reaction (PCR) amplification and reverse hybridization. These steps were carried out in three separate rooms with restricted access and unidirectional workflow. Mycobacterial DNA was extracted in BSL-3 laboratory according to manufacturer's instructions. Briefly, 500 µl of decontaminated sample was centrifuged at 13000 rpm for 15 mins, the supernatant was discarded and the pellet was resuspended in 100 µl lysis buffer. The specimen was then heat killed at 95°C for 5 minutes in Heating Block. This was followed by addition of 100 µl Neutralization buffer and vortex for 5 seconds. The sample was then centrifuged at 13000 rpm for 5 minutes. 5 µl of the DNA supernatant was used for PCR while the remainder was stored at −20°C. Master mixture for amplification consisted of 35 µl, Amplification mix B and 10 µl Amplification mix A (provided with kit) 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 20 cycles comprising denaturation at 95°C for 30 sec and 65°C for 2 min. This was followed by 30 cycles comprising 95°C for 25 sec, 50°C for 40 sec and 70°C for 40 sec and a final extension at 70°C for 8 min. Hybridization was performed with Twincubator. After hybridization and washing, strips were removed, fixed on paper and results were interpreted. Each strip of LPA had 27 reaction zones (bands), including six controls (conjugate, amplification, *Mycobacterium tuberculosis* complex (TUB), *rpoB*, *katG* and *inhA* controls), eight *rpoB* wild-type (WT1–WT8) and four mutant probes (*rpoB* MUT1 D516V, *rpoB* MUT 2A H526Y, rpo*B* MUT2B H526D, and *rpoB* MUT3 S531L), one *katG* wild-type and two mutant probes (*katG* MUT1 S315T1 and *katG* MUT2 S315T2), and two *inhA* wild type and four mutant probes (*inhA* MUT1 C15T, *inhA* MUT2 A16G, *inhA* MUT3A T8C, *inhA* MUT3B T8A) (Figure 1). Either missing of wild-type band or the presence of mutant band was taken as an indication of a resistant strain. Incomplete amplification of RIF and/or INH genes was considered as an invalid result. *Mycobacterium tuberculosis* H37Rv strain was used for quality control purpose.

Informed consent was not required in this study since it was part of routine investigations done in the department. The study was approved by the hospital ethics committee.

RESULTS

Out of the 84 clinical specimens tested, 21 showed acid fast bacilli on ZN staining smear microscopy. All the AFB positive specimens gave a valid result for *Mycobacterium tuberculosis* complex (18 smear positive sputum specimens and 3 BAL) in MTBDR*plus* assay (Table 1). Out of them, 16 were found to be susceptible to both Rif and INH, 5 were resistant to both Rif and INH; without any mono-resistance pattern. All the five MDR-TB specimens were sputum and showed mutation band in both *rpo*B and *kat*G; none in *inh*A. The pattern of mutation showed in Table 2. Among the MDR-TB cases detected, two were newly diagnosed; two completed treatment; one was in continuation phase of anti-tuberculosis therapy.

Among the 63 AFB negative specimens, 12 gave valid result for *M. tuberculosis* complex on MTBDR*plus* assay; sputum (8), BAL (2) and pleural fluid (2).

DISCUSSION AND CONCLUSION

In the present study, GenoType MTBDR*plus* Version 2.0 line probe assay was performed for rapid detection of multidrug resistant *M. tuberculosis* directly from pulmonary samples irrespective of the smear microscopy results. The assay is easy to perform and was successful in rapid detection of MDR TB.

Many recent studies have demonstrated the feasibility of GenoType MTBDR*plus* Line Probe Assay as an effective tool in early detection of MDR TB.[5,6,7]

Table 1: Results of Line Probe Assay and smear microscopy.

Specimen		Smear for AFB		Line Probe Assay for MTB complex	
Type	**Total number**	**Positive**	**Negative**	**Positive**	**Negative**
Sputum	48	18	30	26	22
B.A.L	19	3	16	5	14
Pleural Fluid	17	0	17	2	15

AFB: acid fast bacilli; MTB: *Mycobacterium tuberculosis* BAL: Bronchoalveolar lavage

Table 2: Mutation of MDR-TB specimens.

Specimen		MTBDR*plus* assay results				
ID	**Type**	**Rifampicin resistance**		**Isoniazid resistance**		
		***rpo*B band**	**Mutation**	***kat*G band**	**Mutation**	***inh*A band**
2015/03/44	Sputum	Mut2A	H526Y	Mut1	S315T1	WT
2015/04/157	Sputum	Mut3	S531L	Mut1	S315T1	WT
2015/09/239	Sputum	Mut3	S531L	Mut1	S315T1	WT
2015/12/320	Sputum	Mut1	D516V	Mut1	S315T1	WT
2015/04/348	Sputum	Mut3	S531L	Mut1	S315T1	WT

WT= All wild-type probes are present

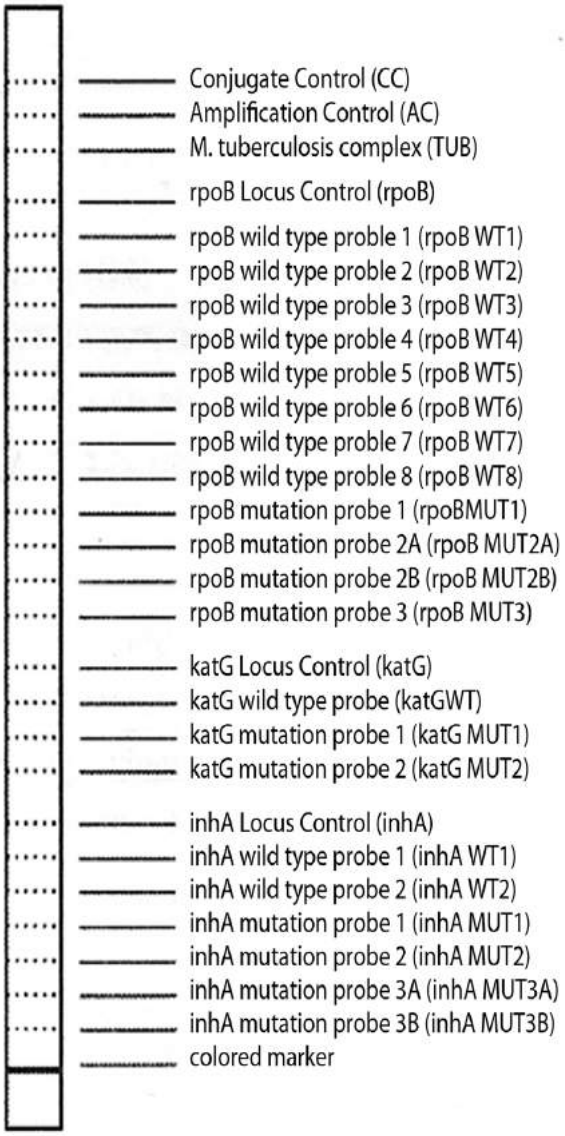

Figure 1: GenoType MTBDR*plus* strip (Hain Lifescience, Nehren,Germany), The strip is not displayed in original size.

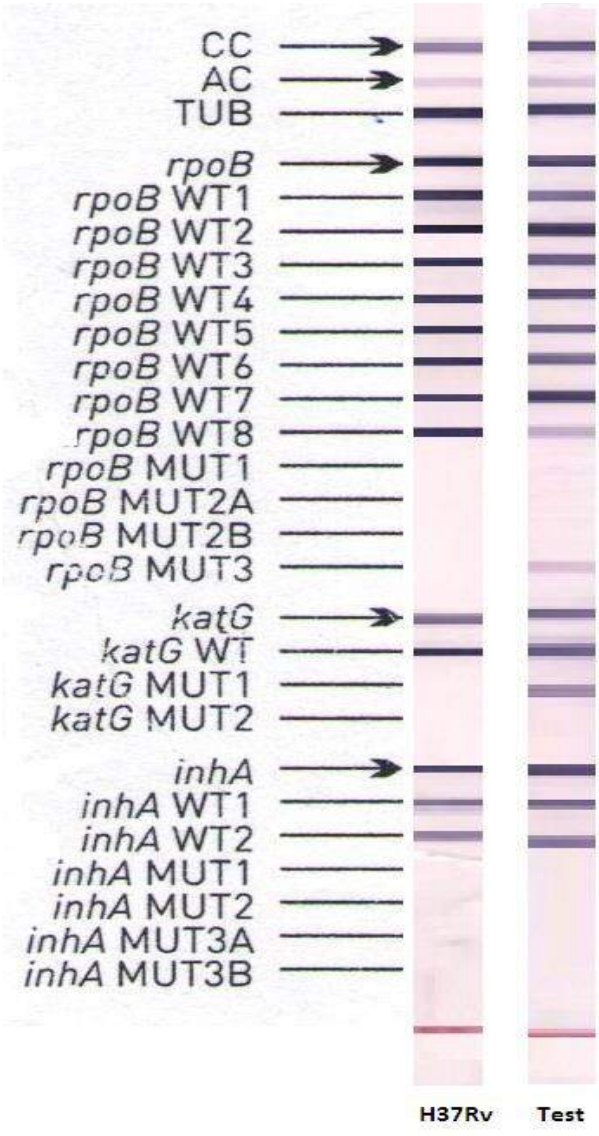

Figure 2: LPA strip showing mutation band in *rpoB* MUT3 and *kat*GMUT1 probe in Test; all bands in WT probes in control (H37Rv) strip.

GeneXpert MTB/RIF test, based on real-time PCR is the most rapid method for the diagnosis of MDR-TB with a turnaround time of approximately two hours where result of rifampicin resistance is used as a surrogate marker of MDR-TB. INH resistance cannot be detected by this method which limits its use for detection of INH monoresistance. Hence, among the available molecular tests, LPA is better than GeneXpert with an additional advantage of detection of INH monoresistance. WHO recommends addition of ethambutol as a third drug in the continuation phase in settings where the level of Isoniazid resistance among new TB cases is high.[8] Additionally, LPA test can also be useful for systematic surveillance of INH monoresistance in countries with high Isoniazid resistance.

Rifampicin resistance is known to be associated with mutations in 81 base pair region (codon 527 to 533) of the *rpo*B gene[9,10,11] The finding of dominant mutation for RIF resistance in *rpo*B S531L [3/5], in the present study is similar to previously published report.[7,12] Distribution of mutations of *kat*G and *inh*A genes is known to vary in different geographical regions. In the present study, all five MDR-TB cases showed *kat*G gene mutation. Neither combined *kat*G and *inh*A gene mutations nor *inh*A gene mutation was detected which may be due to lesser number of specimen of the study.

Though WHO recommendation for use of GenoType MTBDR*plus* VER 2.0 line probe assay for smear negative respiratory specimens is not available, yet the present study demonstrated its utility in detecting MDR-TB in such smear negative specimen.

The limitations of the present study are - failure to compare with conventional culture and DST results and lesser number of clinical specimens to prove the utility of this assay in our geographical region.

To conclude, the GenoType MTBDR*plus* (version 2.0) LPA has the potential to improve the turnaround time for diagnosis of TB and drug resistance in both smear-positive and smear-negative specimens. Additionally, this test also detects monoresistance to Isoniazid and Rifampicin which will aid clinicians to manage cases with MDR TB.

ACKNOWLEDGEMENT

We acknowledge Dr. Pranjal Deuri, Registrar, Department of Pulmonary Medicine, Assam Medical College & Hospital for the clinical assistance during the study. We also acknowledge Department of Biotechnology, Govt. of India and Mr. Lakhi Gogoi, Laboratory technician.

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Source of Support: Nil.

Conflict of Interest: None Declared.

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Cite this article as: Gargi Choudhury, Partha Pratim Das, Lahari Saikia, Nagen Tairai. Early Detection of Multidrug Resistant Tuberculosis Using Genotype MTBDR*plus* Line Probe Assay in a Tertiary Care Centre, Assam.. Int J Med Res Prof. 2016, 2(2); 40-43.