Evaluation of Geno Type MTBDR*plus* Line Probe Assay for Early Detection of Drug Resistance in Tuberculous Meningitis Patients in India

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ABSTRACT

Background: Molecular methods which allow for rapid and reliable detection of drug resistance have yet not been sufficiently evaluated for timely management of patients with tuberculous meningitis. **Aims:** We aimed to evaluate Geno Type MTBDR*plus* line probe assay for early detection of drug resistance in *Mycobacterium tuberculosis* isolates and CSF samples of confirmed tuberculous meningitis patients. **Settings and Design:** This was a multicentric prospective study carried out from July 2011 to December 2013 in tertiary care hospitals of Delhi. **Materials and Methods:** The assay was performed on 89 *M. tuberculosis* isolates and 31 direct CSF samples from microbiologically confirmed tuberculous meningitis patients. The sensitivity and specificity of this assay was calculated in comparison to drug susceptibility testing by BACTEC MGIT 960 system. **Results:** The sensitivity, specificity for detection of resistance to Isoniazid was 93%, 97% and to Rifampicin was 80%, 98.8%, respectively by this assay in comparison with the phenotypic drug susceptibility testing. The line probe assay could detect *M. tuberculosis* in 55% of CSF samples from patients with microbiologically confirmed tuberculous meningitis. Only 5/89 isolates (5.6%) were resistant to both Isoniazid and Rifampicin while 9/89 (10%) isolates were additionally resistant to Isoniazid. Resistance to any of the drugs, namely Isoniazid, Rifampicin, Streptomycin or Ethambutol, was seen in 24.7% of strains. **Conclusion:** The line probe assay has a good sensitivity and specificity for detection of drug resistance to Isoniazid and Rifampicin in *M. tuberculosis* culture isolates. However, this assay has limited role in detection of *M. tuberculosis* and drug resistance from direct samples with confirmed diagnosis of tuberculous meningitis.

Key words: Cerebrospinal fluid (CSF), Geno type MTBDR*plus*, Multidrug-resistant tuberculosis (MDR TB), Tuberculous meningitis (TBM)

BACKGROUND

Tuberculous meningitis (TBM) is one of the most devastating clinical manifestations of tuberculosis with distressing levels of neurological morbidity. The disease is associated with very high mortality (30%) in cases of infection with fully sensitive organism and much higher mortality and morbidity in infection with drug-resistant organisms.[1] The burden of drug resistance in TBM patients is largely unknown in India, but it is believed that drug resistance is increasing due to improper detection, inappropriate treatment and inadequate monitoring and control.[2] It has been estimated that India and China account for nearly 50% of the global burden of multidrug-resistant (MDR) cases with India perhaps having the highest number.[2] Therefore, precise and rapid diagnosis of drug resistance in useful clinical time frame is of utmost importance not only for evidence informed clinical management of TBM but also for reducing spread of MDR tuberculosis.

Conventional culture and drug susceptibility testing on solid/automated liquid culture systems is a time consuming process and suffers from higher contamination rates. The World Health Organization, Geneva and Foundation for

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Innovative New Diagnostics (FIND) have recommended the use of molecular line probe assay (Genotype® MTBDR plus assay, HAIN Life Sciences, Germany) for direct detection of MDR *Mycobacterium tuberculosis* from smear positive sputum specimens and *M. tuberculosis* cultures.[3] This test is based on multiplex PCR followed by reverse hybridization to detect any deletion in wild type gene loci and mutation in rpoB (RNA polymerase B subunit), katG (catalase peroxidase) and inhA (inoyl coenzyme A reducatse) loci.[3]

Though the performance of Genotype® MTBDR plus line probe assay (LPA) has been evaluated in smear positive sputum samples and *M. tuberculosis* (MTB) isolates from pulmonary TB but the assay has not been sufficiently evaluated on MTB culture isolates and direct cerebrospinal fluid (CSF) samples of microbiologically confirmed TBM patients. Early detection of *M. tuberculosis* and MDR TB directly from clinical sample will be of significant help in precise, timely and specific management of this devastating disease.

The present study aimed to:

1. Compare the performance of commercially available Geno Type® MTBDR plus line probe assay (LPA) with automated liquid culture method in BACTEC MGIT 960 (Becton Dickinson, Sparks, MD) for detection of antitubercular drug resistance;
2. Evaluate the utility of this line probe assay for detection of MTB and drug resistance directly from CSF samples of microbiologically confirmed TBM patients;
3. Determine the magnitude and molecular profile of drug resistance in TBM patients.

MATERIALS AND METHODS

This prospective study was a collaborative project conducted over a period of 30 months in department of Microbiology of Institute of Human Behaviour and Allied Sciences (IHBAS), Delhi, with support from department of Neurology, IHBAS, GB Pant Hospital, Guru Teg Bhahdur Hospital, and Chacha Nehru Bal Chikitsalaya, Delhi, India. The patients were enrolled from July 2011 to December 2013 from IHBAS and from July 2012 to December 2013 from all other centers. Ethical approval for the study was obtained from their respective ethics committee. Informed written consent was obtained from all patients involved in the study.

A total of 238 suspected cases of TBM who were not started on anti-tubercular treatment (ATT), were enrolled in the study following criteria laid down by Marias *et al*.[4] Approximately 1-5 ml of CSF sample was received from each patient in mycobacteriology laboratory for microbiology work-up. The cellular and biochemical analysis of CSF were done in laboratories of their respective institutes. After separating 1 ml of sample for microscopy and culture, any remaining sample was stored at −20˚C for further molecular analysis.

Culture and DST on BACTEC MGIT 960 system

CSF was cultured both by conventional (Lowenstein Jensen solid culture media) and automated (BACTEC MGIT 960 system) methods following standard microbiological techniques.[5,6] A 500 µl aliquot of CSF was directly inoculated in BACTEC MGIT 960 tubes as per manufacturer's instructions and the remaining sample was centrifuged at 3000 g for 10 min and used for smear examination and conventional culture. Any positive signal or growth was subjected to smear examination and confirmation for MTB by Para Nitro Benzoic acid test (PNB) and Immuno-chromatographic test (TB Ag MPT64, SD, Germany). Drug susceptibility testing (DST) was done using Streptomycin (1 µg/ml), Isoniazid (INH) (0.1 µg/ml), Rifampicin (RIF) (1 µg/ml), and Ethambutol (5 µg/ml) as per FDA-approved method in BACTEC MGIT 960 for all positive cultures.[6] The molecular profile for INH and RIF resistance was determined by the Geno Type MTBDRplus Assay as described.[7]

Genotype MTBDRplus assay

DNA was extracted using Genolyse kit (HAIN Life Sciences, Germany). Multiplex PCR for the detection of drug resistance genes (rpoB, katG, inhA) was performed using 35 µl of primer nucleotide mix, 10 µl of Taq DNA polymerase-PCR buffer mix and 5 µl of supernatant in a final volume of 50 µl. Amplification was done in a thermal cycler (PalmCycler, Genetix Biotech Asia Pvt. Ltd) using cycling parameters as: Initial denaturation of 15 min at 95˚C, followed by 20 cycles of 30 sec at 95˚C and 2 min at 65˚C, and 30 cycles of 25 sec at 95˚C, 40 sec at 50˚C and 40 sec at 70˚C and the extension step of 8 min at 70˚C. Reverse hybridization was performed using Twincubator (HAIN Life Sciences, Germany) by Geno Type MTBDRplus kit as per manufacturer's instruction to find out any deletion in wild-type gene loci and mutation in rpoB (RNA polymerase B subunit), Kat G (catalase peroxidase) and inhA (inoyl coenzyme A reducatse) loci.

The hybridization strips consist of 27 reaction zones (6 control probes and 21 probes for mutation). The control probes include a conjugate control, amplification control,

M. tuberculosis complex control (TUB), rpoB amplification control, inhA amplification control and katG amplification control. For the detection of rifampicin resistance, the rpoB gene (coding for the β-sub-unit of the RNA polymerase) and for high level INH resistance, the katG gene (coding for the catalase peroxidase) is examined and for detection of low-level INH resistance, the promoter region of the inhA gene (coding for the NADH enoyl ACP reductase) is examined. For a valid result, all the 6 control bands should appear correctly. The absence of any of the wild-type bands and/or presence of any mutation band implies resistance to the particular antibiotic tested [Figure 1].

PCR for *M tuberculosis*

DNA was extracted using QIAamp DNA mini kit.[8] Nested PCR was done for amplification of mpt64 gene in MTB [NCBI Reference Sequence: NC_000962.3] using the outer forward primer 5'-ATCCGCTGCCAGTCGTCTTCC-3' and outer reverse primer 5'-TCGCGAGTCTAGGCCAGCAT-3' as initial denaturation at 96˚C for 3 min followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 1 min. A final extension was done at 72˚C for 10 min. Second round of PCR was done using inner forward primer 5'-GTGAACTGAGCAAGCAG-3' and inner reverse primer 5'-GTTCTGATAATTCACCGG-3' as: Incubation at 50˚C for 2 min and initial denaturation at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 1 min. Amplified gene product was analyzed using agarose gel (2.5%) electrophoresis.

Genotype MTBDR plus assay on direct CSF samples

Representative microbiologically confirmed TBM CSF samples (5 smear positive, 16 culture positive, smear negative, and 10 PCR positive, smear negative and culture negative) were subjected to DNA extraction retrospectively by Genolyse kit (HAIN Life Sciences, Germany). Rest of the procedure was same as described in the Geno Type MTBDRplus Assay.

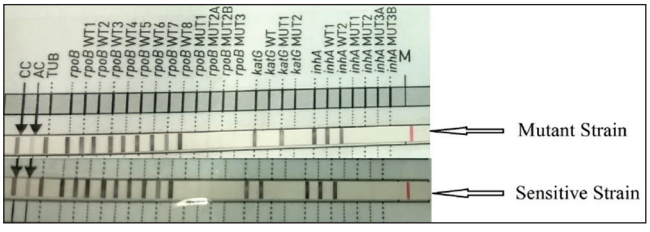

Figure 1: Geno Type MTBDR*plus* assay for mutant (resistant) and wild (sensitive) *Mycobacterium tuberculosis* strains

RESULTS

Out of 238 clinically suspected TBM cases, only 5 samples were positive by smear microscopy and 89 samples grew MTB in culture (including smear positive samples). Of these, 78 samples grew MTB only in BACTEC MGIT 960, 2 samples only in conventional culture and 9 samples grew MTB in both BACTEC and conventional culture.

All the 89 MTB isolates were subjected to both MGIT DST and LPA. Table 1 depicts results of drug susceptibility testing by BACTEC MGIT 960. Five strains were identified as MDR TB (resistant to INH and RIF) by BACTEC MGIT 960 but only four strains were found to be MDR TB by both MGIT DST and LPA. DST in BACTEC MGIT 960 identified nine more strains resistant to INH, seven only to INH and two more to INH and streptomycin. Overall, concordance between results of MGIT DST and LPA for INH and RIF was seen in 84/89 isolates (94.3%). Discordant results in MGIT DST and LPA were present in only five isolates, three for INH resistance and two for Rifampicin resistance. For INH, two strains were resistant by LPA and susceptible by MGIT DST while one strain was susceptible in LPA and resistant in MGIT DST. For Rifampicin, one strain was resistant in MGIT DST while susceptible in LPA and one strain had vice versa results [Table 2]. Considering 1% phenotypic proportion method in BACTEC MGIT as the gold standard, sensitivity and specificity of Genotype MTB DR assay for detection of resistance to INH was 93% and 97% with PPV of 86.6%

Table 1: Results of drug susceptibility testing by BACTEC MGIT 960 (*n* = 89)

Resistance to	By BACTEC MGIT 960 system
Only INH	7
Only RIF	Nil
Only STR	5
Only ETH	3
INH+RMP	4
INH+RMP+STR	1
INH+STR	2

INH: Isoniazid, RIF: Rifampicin, STR: Streptomycin, ETH: Ethambutol

Table 2: Performance of genotype® MTBDR assay in MTB isolates against conventional MGIT DST

Genotype® MTBDR assay	BACTEC MGIT drug susceptibility testing			
	$INH^R RIF^R$	$INH^R RIF^S$	$INH^S RIF^R$	$INH^S RIF^S$
$INH^R RIF^R$	4	1	—	—
$INH^R RIF^S$	1	7	—	2
$INH^S RIF^R$	—	—	—	—
$INH^S RIF^S$	—	1	—	73

INH: Isoniazid, RIF: Rifampicin, S: Sensitive, R: Resistant

and NPV of 98.6%. Sensitivity and specificity for detection of resistance to RIF was 80% and 98.8% with PPV of 80% and NPV of 98.8% [Table 3].

LPA from direct samples

Table 4 depicts the performance of LPA on 31 direct clinical samples. *M. tuberculosis* control band (TUB) was absent in 14 samples so overall sensitivity of LPA for detection of MTB and drug resistance was only 55% (17/31). However, molecular profile of drug resistance in 10 culture-positive CSF samples was well matched with respective MTB culture results.

Molecular drug resistance profile

Molecular profile of 94 samples was available for analysis as five additional PCR positive samples gave valid interpretable results in LPA. Out of the five RIF-resistant strains by LPA, deletion in the 81 bp region (WT8 band) was observed in two strains, mutation in rpoB S531L (MUT 3 band) in two strains and deletion of WT8 and WT6 band in one strain. In 17 INH-resistant strains, katG mutation S315T1 region (MUT1 band) depicting high level resistance to INH was detected in 15 (12 culture isolates+3 clinical samples) strains while mutation in inhA C15T region depicting low level resistance to INH was detected in two strains. Additional deletion of wild type band (WT) was seen in 10/15 (67%) strains with katG mutation in S315T1 region (MUT1 band). All the MDR isolates had high-level resistance to INH [Table 5].

Table 3: INH and RIF resistance by Geno Type MTBDR*plus* assay compared with BACTEC MGIT 960 DST

Genotype MTBDR plus (*n* = 89)	BACTEC DST (*n* = 89)	
	Resistant	Susceptible
INH resistant (15)	13	2
INH sensitive (74)	1	73
RIF resistant (5)	4	1
RIF sensitive (84)	1	83

Table 4: Performance of the Geno Type MTBDR*plus* assay in microbiologically confirmed CSF samples

Geno Type MTBDR*plus* assay	Smear +ve	Culture +ve, smear –ve	PCR +ve, smear –ve and culture –ve
Positive	2	10	5
Negative	3	6	5
Total	5	16	10

Assay positive: Presence of all six control bands, Assay negative: Absence of MTB control band

Table 5: Molecular profile of drug-resistant MTB strains from culture isolates and clinical samples

ID	Rifampicin		Isoniazid		
	rpoB Pattern	DST	katG Pattern	inhA Pattern	DST
1268	—	R	ΔWT, S315T1	—	R
1922	—	S	ΔWT, S315T1	—	R
1926	S531 L	R	S315T1	—	R
2068	S531 L	R	ΔWT, S315T1	—	R
2150	—	S	ΔWT, S315T1	—	R
2175	ΔWT8	R	ΔWT, S315T1	—	R
2308	—	S	ΔWT, S315T1	—	R
2364	ΔWT6, ΔWT8	R	S315T1	—	R
2421	—	S	—	C15T	S
2486	—	S	ΔWT, S315T1	—	R
2618	—	S	ΔWT, S315T1	—	R
2658	—	S	ΔWT, S315T1	—	R
2383	ΔWT8	S	—	—	S
2398	—	S	ΔWT, S315T1	—	R
1832	—	S	—	C15T	S
2472	—	S	—	—	R
2804	—	ND	S315T1	—	ND
2824	—	ND	S315T1	—	ND
3059	—	ND	S315T1	—	ND

S: Sensitive, R: Resistant, – Wild type (no resistance), ΔWT: Deletion wild type, ND: Not done

DISCUSSION

Early confirmed diagnosis of TBM with rapid detection of drug resistance is highly desirable for timely effective management of this medical emergency. For this purpose, a commercially available LPA (Genotype® MTBDR plus assay) was evaluated for its usefulness on culture isolates and direct CSF samples of TBM patients. This study has addressed the utility of this molecular assay on culture isolates of MTB as well as direct CSF samples from TBM patients.

The user friendliness and ease of performance of this test within one working day is well documented in many studies and we also had the same experience.[9] Using BACTEC MGIT DST as a gold standard, the sensitivity and specificity of LPA for INH resistance was 93%, 97% and for RIF resistance 80%, 98.8%, respectively. In comparison, evaluation of this assay from China in MTB isolates of TBM patients has demonstrated 100% concordance in MGIT DST and LPA results.[10] This could have been due to fewer numbers of MTB isolates tested by the line probe assay and phenotypic DST.[10] However, results of our study are in good agreement with previously reported studies in MTB pulmonary isolates.[9,11,12] Thus, the assay

demonstrated good concordance with the automated liquid culture-based system for detection of drug resistance in MTB culture isolates from TBM patients. A sensitivity of 93% for INH in this study suggested that most of the mutations conferring INH resistance in MTB CNS isolates are also present in the gene region incorporated in LPA. However, slightly lower sensitivity for RIF could probably be due to low absolute number of resistant strain.

There was discordance observed in three MTB strains for INH. Two strains showed sensitivity to INH by MGIT DST but low level resistance (mutation in inhA 15 region) by LPA. This resistance could have been missed in phenotypic assay because of the use of higher concentrations of INH which could have inhibited the growth of MTB, thus susceptible results.[9] Only one strain was detected resistant by MGIT DST and susceptible by LPA, this could have been due to unidentified mutation in some other genomic region (like ahpc, kasA, furA) which are not targeted by this assay.[12] For RIF resistance there were only two discordant results. One isolate was resistant to RIF by LPA but showed susceptibility by MGIT DST. This isolate had only deletion of WT8 band. This could be false positive as studies have shown absence of only WT8 band should not be considered resistant unless correlated with phenotypic results.[11] One isolate was detected resistant to Rifampicin by MGIT DST but sensitive by LPA. This could be because of some rare mutations occurring outside the 81bp region of the *rpoB* gene which are not targeted in this assay.[12]

In this study, we could detect MTB and drug resistance using LPA in only 55% of confirmed TBM patients from direct CSF. These results are in contrast to results reported by Liu *et al*., who could detect MTB and drug resistance in 28 of the 30 PCR-positive clinical samples.[10] Our study could not detect MTB in 14 of the 31 confirmed TBM samples. This is surprising as it is generally accepted that lower limit for detection of acid fast bacilli in smear microscopy is 10^4-10^5 bacilli/ml, in culture 10^3-10^2 bacilli/ml and in PCR 1-10 bacilli/ml and thus LPA should have detected MTB in these direct samples. Therefore, this assay needs to be further evaluated for its utility in a larger cohort of direct CSF samples.

Using MGIT DST as gold standard, 75.3% of all isolates (67/89) were sensitive to four primary drugs. Twenty-two of 89 isolates (24.7%) had resistance to at least any of the four drugs tested. The prevalence of MDR (resistance to INH and RIF) was seen in 5.6% of isolates and one of these MDR strain had additional resistance to streptomycin. Mono resistance to INH, Streptomycin or Ethambutol was observed in 7.8%, 5.6% and 3.4%, respectively. Monoresistance to Streptomycin or Ethambutol does not seem to be problematic in treatment of TBM patients but resistance to INH is worrisome as INH is a potent bactericidal drug capable of freely penetrating CSF and INH resistance is a strong predictor of death in patients with TBM.[13,14] The lower prevalence of drug resistance for MTB in this study was found to be almost similar to other studies from India and is therefore, not yet, a serious threat in TBM as compared to that with pulmonary TB [Table 1].[15,16]

Regarding molecular basis of drug resistance all but two INH-resistant strains had mutation in katG S315T1 gene loci. Ten of these strains had additional deletion of WT gene loci. Two strains had low level resistance to INH with a mutation in inhA C15T gene loci. None of the strains tested had mutation in katG and inhA gene simultaneously. For RIF the most common mutations was seen in 81 base pair region of the rpo B gene (codon 530-533 and/or codon 518-525). In only one strain there was deletion of only WT8 band. Molecular profile of TBM isolates indicate that similar genotypic clusters exist among pulmonary and meningeal tuberculosis in India.[9,11]

CONCLUSION

The overall prevalence of MDR (resistance to INH and RIF) in MTB isolates from patients of TBM was found to be 5.6% with additional INH resistance of the order of 10%. In this multicenteric study from Delhi, India, Geno Type® MTBDR*plus* assay was proved to be a sensitive and specific tool for detection of drug resistance from MTB isolates of TBM patients and is capable of detecting both INH and RIF resistance. This assay can also detect low-level resistance to INH. As INH resistance is a strong predictor of patient outcome its early detection becomes crucial for patient management. In our experience, the much lower sensitivity of this assay for detection of MTB in direct CSF samples is a cause for some concern. Therefore, it is recommended that all the culture positive isolates of TBM should be immediately subjected to Geno Type® MTBDR plus assay and drug resistance results be communicated without waiting for MGIT DST results. Where resources are not the constrain, direct CSF samples which are smear or PCR positive can also be subjected to this line probe assay for detection of drug resistance as about half the patients may still immensely benefit from early and specific detection of drug resistance in TBM.

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