Indian Journal of Medical Microbiology, (2013) 31(3): 230-236

Original Article

Use of GenoType® MTBDR*plus* assay to assess drug resistance and mutation patterns of multidrug-resistant tuberculosis isolates in northern India

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Abstract

Purpose: The emergence and spread of multidrug-resistant tuberculosis (MDR-TB) is a major public health problem. The diagnosis of MDR-TB is of paramount importance in establishing appropriate clinical management and infection control measures. The aim of this study was to evaluate drug resistance and mutational patterns in clinical isolates MDR-TB by GenoType® MTBDR*plus* assay. **Material and Methods:** A total of 350 non-repeated sputum specimens were collected from highly suspected drug-resistant pulmonary tuberculosis (PTB) cases; which were processed by microscopy, culture, differentiation and first line drug susceptibility testing (DST) using BacT/ALERT 3D system. **Results:** Among a total of 125 mycobacterium tuberculosis complex (MTBC) strains, readable results were obtained from 120 (96%) strains by GenoType® MTBDR*plus* assay. Only 45 MDR-TB isolates were analysed for the performance, frequency and mutational patterns by GenoType® MTBDR*plus* assay. The sensitivity of the GenoType® MDRTB*plus* assay for detecting individual resistance to rifampicin (RIF), isoniazid (INH) and multidrug resistance was found to be 95.8%, 96.3% and 97.7%, respectively. Mutation in codon S531L of the *rpoB* gene and codon S315T1 of *katG* genes were dominated in MDR-TB strains, respectively ($P < 0.05$). **Conclusions:** The GenoType® MTBDR*plus* assay is highly sensitive with short turnaround times and a rapid test for the detection of the most common mutations conferring resistance in MDR-TB strains that can readily be included in a routine laboratory workflow.

Key words: *GenoType® MTBDRplus assay, MDR-TB, M. tuberculosis complex, tuberculosis*

Introduction

The worldwide emergence of multidrug-resistance tuberculosis [(MDR-TB), i.e., resistance to at least rifampicin (RIF) and isoniazid (INH)] in association with human immunodeficiency virus (HIV) infection is continuously increasing.[1] The prevalence of MDR-TB among new and previously treated cases is increasing all over the world as well as in India.[2,3] The transmission of drug-resistant tuberculosis (DR-TB) strains is increasing because of the growing burden of MDR-TB patients.[2,4] Rapid detection of MDR-TB allows the establishment of an effective treatment regimen; minimises the risk of further resistance and limits spread of drug-resistant strains.[5] During the past few years, several molecular techniques have been developed, including conventional sequencing, pyrosequencing, real time polymerase chain reaction (PCR) and reverse hybridisation assay with DNA probes. These molecular techniques have been proposed for detection of mutation frequency and patterns associated with drug resistance with the later methodology in a number of in-house and commercial assays.[6-9] In June 2008, the World Health Organisation (WHO) recommended the use of molecular line probe assay for the diagnosis of MDR-TB.[10] The Genotype® MTBDR*plus* assay, a commercially available multiplex PCR DNA strip assay (Hain Lifescience, Nehren, Germany), is designed to simultaneously detect the most important *rpoB* and *katG* gene mutations conferring RIF and high-level INH resistance in the clinical isolates.[9] It is based on the principle of multiplex PCR in combination with reversed hybridisation to identify *rpoB* and *katG* amplicons to membrane-bound probes. The DNA strip covers eight *rpoB* wild-type probes, four *rpoB* mutant probes (with D516V, H526Y, H526D and S531L mutations), one *katG* wild-type probe and two *katG* mutant probes (with S315T1 and S315T2 mutations).[11] The mutations that predominate in RIF-resistant *Mycobacterium tuberculosis* isolates are located in an 81-bp "core region" of the *rpoB* gene (95% of all RMP-resistant strains).[11-13] Resistance to INH conferred by mutations in catalase–peroxidase enzyme gene (50-95% of INH-resistant strains) is targeted in codon 315 of the *katG* gene[14-16] and 20-35% contain mutations in the *inhA*

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Received: 18-02-2013
Accepted: 28-05-2013

Access this article online	
Quick Response Code:	Website: www.ijmm.org
	DOI: 10.4103/0255-0857.115625

regulatory region and an additional 10-15% have mutations in the *ahpC-oxyR* intergenic region.[15-17] The aim of this study was to assess drug resistance and mutational patterns in clinical isolates of MDR-TB by GenoType® MTBDR*plus* assay.

Materials and Methods

Study design and setting

This was a prospective, hospital-based clinico-microbiological observational study conducted at a two tertiary-level hospital in Lucknow, India. This study was conducted after approval by the local research ethics committee. Informed consent was obtained from the patients for sample collection and enrolment in this study.

Patient selection

Suspected DR-TB patients referred for evaluation and management from January 2011 to July 2012 were enrolled in this study. Approximately 2-10 ml of non-repeated sputum specimens, bronchial washing, bronchial lavage and other pulmonary samples were collected from pulmonary tuberculosis (PTB) patients. The present, past and family history of TB or anti-tubercular treatment (ATT) and any other associated chronic diseases were evaluated in the prescribed proforma.

Criteria for inclusion

Patients included in the study were new or previously treated suspected DR-TB cases from all age groups, in whom TB was confirmed by culture and in whom drug susceptibility testing (DST) against *M. tuberculosis* complex strains had been performed. Those infected with mycobacteria other than tuberculosis (MOTT), patients with an unknown bacteriological profile and those patients not willing to participate were not included in the present study.

Processing, smear and culture of clinical specimens

All the clinical specimens received were subjected to direct smear microscopy by Ziehl–Neelsen (ZN) staining method.[18] Specimens, which contain normal commensal bacterial flora, were decontaminated by the standard N-acetyl-L-cysteine-NaOH method.[19] Specimens were centrifuged and the sediment was inoculated into the vials of the BacT/Alert 3D system (bioMerieux, France) containing modified Middlebrook 7H9 with an antibiotic supplement (amphotericin B [0.018%, wt/vol], azlocillin [0.0034%, wt/vol], nalidixic acid [0.04%, wt/vol], trimethoprim [0.00105%, wt/vol], polymyxin B [10,000 U] and vancomycin [0.0005%, wt/vol]). BacT/Alert 3D vials were monitored continuously by the BacT/Alert 3D system.[20] Positive vials were subjected to smear microscopy for the presence of acid fast bacilli (AFB). No growth after 6 weeks of incubation was treated as negative for mycobacteria. Positive cultures for *M. tuberculosis* complex were typed by niacin production, catalase activity at 68°C and pH 7 and susceptibility to p-nitrobenzoic acid.[21]

Identification of M. tuberculosis complex strains

The identification and differentiation of mycobacterium tuberculosis complex (MTBC) strains from non-tuberculous mycobacteria (NTM) were performed by the GenoType® CM assay as per the manufacturer's instructions (Hain Lifescience GmbH, Germany).[22] The standard strain of M. tuberculosis complex, H37 *Rv* ATCC™ No. 27294, was used as positive control.

BacT/ALERT drug susceptibility testing by standard 1% proportion method

The BacT/ALERT MB susceptibility reagents and the glass BacT/ALERT MP (Mycobacteria Process) bottles were procured from bioMerieux, France. DST was performed as per the manufacturer's protocol.[20] Briefly, 0.5 ml of the lyophilised antibiotic solutions and 0.5 ml restoring fluid were added to the glass BacT/ALERT MP test bottles and the undiluted direct control bottle, respectively. The final drug concentrations in the test bottles were 0.9 mg/l for RIF, 0.4 mg/l for INH. A total of 0.5 ml of the seeded inoculum was added to all BacT/ALERT MP test bottles. Bottles were loaded into the BacT/ALERT 3D system simultaneously, and the maximum test time was automatically limited to 15 days. The same standard strain of *M. tuberculosis complex*, H37 *Rv* ATCC™ No. 27294, was used as positive control.

GenoType® MTBDRplus assays

All culture positive MTBC clinical isolates were subjected to first line anti-tubercular resistance, frequency and mutational analysis by a GenoType® MTBDR*plus* assay as per manufacturer's instructions. Briefly, for amplification 35 µl of a primer-nucleotide mixture (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U hot start Taq polymerase (QIAGEN, Hilden, Germany) and 5 µl of a preparation of chromosomal DNA in a final volume of 50 µl were used. 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. Reverse hybridisation and detection was performed in an automated washing and shaking device (Profiblot; Tekan, Maennedorf, Switzerland). The hybridisation procedure was performed at 45°C for 0.5 hours, followed by washing steps and the colorimetric detection of the hybridised amplicons. After a final wash, the strips were air dried and fixed on paper provided with the kit.

The GenoType® MTBDR*plus* strip contains 17 probes, including amplification and hybridisation controls to verify

the test procedures. For the detection of RMP resistance, eight *rpoB* wild-type probes (probes WT1 to WT8) encompass the region of the *rpoB* gene encoding amino acids 509 to 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 RIF. For the detection of 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. Furthermore, 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. Again, either the absence of one or more wild-type probe (s) or the presence/staining of a mutant probes were indicative of resistant strain as shown in Figure 1.

Data analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of GenoType® MTBDR*plus* assay results were compared with the conventional BacT/ALERT 1% proportion DST results for RIF, INH and MDR-TB. An analysis of the frequency and mutational patterns associated with MDR-TB strains were performed by using the GenoType® MTBDR*plus* assay. Data were analysed using SPSS 15.0 (Statistical Package for the Social Sciences, Chicago, IL, USA) for Windows. For comparison of data, a value less than 0.05 were considered statistically significant.

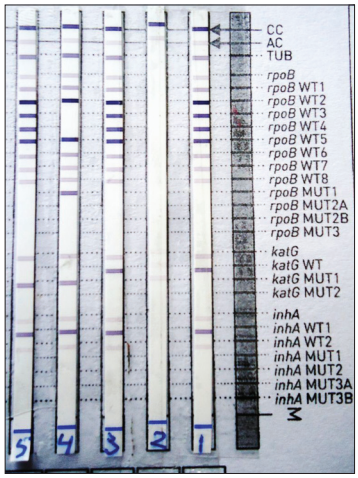

Figure 1: Representative DNA strip patterns obtained with the GenoType® MTBDR*plus* assay. The positions of the oligonucleotide probes are given on the right. The target genes and specific probe lines are shown from the top to bottom as follows: Conjugate control; amplification control (23S rRNA); *M. tuberculosis* complex-specific control (23S rRNA); locus control of *rpoB* amplification; eight *rpoB* wild-type (WT) probes; four *rpoB* mutant probes with mutations in codons 516, 526 or 531; locus control of *katG* amplification; one *katG* codon 315 WT probe; two *katG* probes with mutations in codon 315; locus control of *inhA* amplification; two *inhA* WT probes; and four mutation probes in the *inhA* promoter region (−15C/T, −16A/G, −8T/C and − 8T/A). Samples with different strip susceptibility patterns are shown on the left. Lane 1, *M. tuberculosis* complex H37Rv control strain (*rpoB, katG, inhA* WT); lane 2, absence of TUB (excluded from the study analysis); Lane 3, fully susceptible strain (*rpoB, katG, inhA* WT); lane 4, multidrug-resistant tuberculosis (MDR-TB) (absence of WT (3,4) and presence of MUT1 D516V in *rpoB*, *katG* presence of MUT1 S315T1 and absence of WT 1; lane 5, INH mono-resistance (presence of *katG* S315T1 and absence of WT)

Results

Smear microscopy, identification and phenotypic drug susceptibility tests by BacT/ALERT 3D culture

Among a total of 350 sputum specimens collected from PTB patients of highly suspected cases of treatment defaulters, re-treatment and relapse cases, only 75 (21.4%) were AFB positive in ZN microscopy and 165 (47.1%) were positive for mycobacteria by BacT/ALERT MP culture. After using a panel of different biochemical and molecular tests; 125 (75.7%) strains confirmed as *M. tuberculosis* complex, and 30 (24.3%) were confirmed as NTM by GentoType® Mycobacterium CM assay. DST by 1% proportion method was performed over only 125 MTBC strains, among which 33 (26.4%) cases were newly diagnosed, and 92 (73.6%) were previously treated TB cases. We found 45 (36%) MDR-TB strains from 125 MTBC isolates tested for first line drugs by BacT/ALERT MP culture; among which a total of 81 (64.8%) strains identified as resistant to one or more than one anti-tubercular drug, and 44 (35.2%) strains were fully susceptible (SS). Single drug resistance (mono-resistance to any drug) was seen only in 22 (17.2%), any two drug resistance in 25 (30.8%), any three drug resistance in 21 (25.9%) and all four drug resistance seen in 13 (16.1%) MTBC strains.

Readable GenoType® MTBDR*plus* assay results were obtained from 120 MTBC isolates, out of a total 125 MTBC culture positive isolates; comprising 96% of all extracts available for analysis and remaining 5 (4%) samples were excluded from the study due to unreadable or having either no bands at all or very light/weak/unreadable bands in *rpoB, katG* and/or *inhA* sections.

Concordance between conventional DST and GenoType® MTBDRplus assay

Performance of GenoType® MTBDR*plus* assay was calculated by comparison with conventional DST results over a total of 120 MTBC isolates tested [Table 1]. Considering the phenotypic proportion DST method as the gold standard, sensitivity for detection of RIF, INH and MDR-TB by GenoType® MTBDR*plus* assay was 95.8%, 96.3% and 97.7%, respectively. The sensitivity of the GenoType® MTBDR*plus* assay for MDR-TB strains was 97.7% (95% CI:

0.88-0.99%); for RIF resistant strains 95.8% (95% CI: 0.85-0.99%) and for INH resistant strains was 96.3% (95% CI: 0.87-0.99%). The specificity for detection of MDR-TB was 99.1% by GenoType® MTBDR*plus* assay. The concordance between conventional DST and GenoType® MTBDR*plus* assay for RIF, INH and MDR-TB are shown in Table 1.

Frequency and mutational patterns of MDR-TB by GenoType® MTBDRplus assay

The frequency and mutational patterns were analysed by GenoType® MTBD*plus* assay are shown in Tables 2 and 3. Among a total of 45 MDR-TB strains, 36 (80%), 42 (93.3%)

Table 1: Correlation with conventional DST and GenoType® MTBDR*plus* assay, CI 95% (*n*=120)

	Rifampicin	Isoniazid	Multidrug resistance
Sensitivity (%)	95.8 (0.85-0.99)	96.3 (0.87-0.99)	97.7 (0.88-0.99)
Specificity (%)	98.5 (0.91-0.99)	98.4 (0.91-0.99)	99.1 (0.95-0.99)
PPV (%)	97.8 (0.88-0.99)	98.1 (0.89-0.99)	97.7 (0.88-0.99)
NPV (%)	97.1 (0.89-0.99)	96.7 (0.88-0.99)	99.1 (0.95-0.99)

PPV: Positive predictive value, NPV: Negative predictive value, DST: Drug susceptibility testing

Table 2: Frequency of mutations in MDR-TB by GenoType® MTBDR*plus* assay (*n*=45)

Resistance gene	Mutation probe	Codons analysis	No. (%)
#*rpoB*	D516V (MUT1), H526Y (MUT2A), H526D (MUT2B), H531L (MUT3)	505-533	36 (80)
#*katG*	S315T1 (MUT1)	315	42 (93.3)
#*inhA*	C15T (MUT1), A16G (MUT2)	–8 to –15	13 (28.9)
Frequency of different mutations in *rpoB*, *katG* and *inhA* gene			
rpoB	D516V (MUT1)	513-519	8 (17.7)
	H526Y (MUT2A)	526-529	6 (11.1)
	H526D (MUT2B)	526-529	1 (2.2)
	H531L (MUT3)	530-533	28 (62.3)
	*Unknown	-	9 (20)
katG	S315T1 (MUT1)	315	42 (93.3)
	*Unknown	-	3 (6.7)
inhA	C15T (MUT1)	-15	8 (17.7)
	A16G (MUT2)	-16	5 (11.1)
	*Unknown	-	32 (71.4)

*Absence of any one or more wild-type (WT), #Excluding absence of any one or more WT

Table 3: Mutational patterns associated with RIF and INH resistance (*n*=45)

rpoB mutations	*katG* mutations	*inhA* mutations	Frequency	Percentage
D516V (MUT1)	S315T1 (MUT1)	Unknown*	4	8.9
H526Y (MUT2A)	S315T1 (MUT1)	C15T (MUT1)	1	2.2
H526D (MUT2B)	S315T1 (MUT1)	Unknown*	1	2.2
H531L (MUT3)	S315T1 (MUT1)	C15T (MUT1)	3	6.7
H531L (MUT3)	S315T1 (MUT1)	Unknown*	17	37.8
D516V (MUT1)+H526Y (MUT2A)	S315T1 (MUT1)	Unknown*	1	2.2
D516V (MUT1)+H526Y (MUT2A)+H531L (MUT3)	S315T1 (MUT1)	C15T (MUT1)	1	2.2
D516V (MUT1)+H531L (MUT3)	S315T1 (MUT1)	Unknown*	1	2.2
H526Y (MUT2A)+H531L (MUT3)	S315T1 (MUT1)	Unknown*	1	2.2
Unknown*	Unknown*	C15T (MUT1)	1	2.2
Unknown*	S315T1 (MUT1)	Unknown*	6	13.3
Unknown*	Unknown*	C15T (MUT1)	2	4.4
D516V (MUT1)+ H526Y (MUT2A)+H531L (MUT3)	S315T1 (MUT1)	Unknown*	1	2.2
H531L (MUT3)	S315T1 (MUT1)	A16G (MUT2)	4	8.9
H526Y (MUT2A)	S315T1 (MUT1)	A16G (MUT2)	1	2.2
Total			45	100

RIF: Rifampicin, INH: Isoniazid

and 13 (28.9%) strains harboured known mutation in *rpoB*, *katG* and *inhA* genes, respectively. The frequency of *rpoB* mutation was 28 in S531L (62.3%), 8 in D516V (17.7%), 6 in H526Y (11.1%), 1 in H526D (2.2%) region and 9 (20%) were unknown mutations (absence of one or more wild-type) ($P < 0.05$). The most prominent mutations in *katG* and *inhA* genes were 42 in S315T1 (93.3%) and 8 in C15T (17.7%) region, respectively ($P < 0.05$). There were some other mutations, which are shown in Tables 2 and 3. We found 37.8% (H531L + S315T1) as the most common mutational pattern, followed by 8.9% (D516V + S315T1), 6.7% (H531L + S315T1 + C15T) and 2.2% (D516V + H526Y + H531L + S315T1 + C15T). There were some other mutational patterns, which are shown in Table 3.

Discussion

In the present study we studied the performance of the GenoType® MTBD*plus* assay for the rapid detection of MDR-TB as well as frequency of different resistance with mutational patterns among clinical isolates from PTB patients in the northern India. The performance of the GenoType® MTBDR*plus* assay was correlated very accurately with DST by 1% proportion method using BacT/ALERT 3D system. The sensitivity for the detection of rifampicin resistance in our study was 95.8%, which was similar to other reports from Germany, Italy, Finland, France, Denmark, Turkey, Vietnam and Taiwan (92-100%, $P > 0.05$).[5,9,23-28] The sensitivity for detection of INH resistance in our study was 96.3%, which was similar to other reports from Germany, Finland, Denmark and Taiwan (84-100%, $P > 0.05$) but higher than reports from Turkey, Italy, France and the Caribbean (35-73%, $P > 0.05$). [5,9,23-29] The sensitivity of GenoType® MTBDR*plus* assay for detection of MDR-TB was 97.7%, which was higher than previous reports from South Africa, Germany, Russia and Italy.[24,27,28] Increasing trends of TB and MDR-TB rates in high TB burden countries require development and implementation of rapid diagnostic techniques and the ability to correctly detect MTBC and MDR-TB in clinical specimens. Phenotypic DST is a time consuming process because it requires culture, which may require 4-6 weeks or a longer time. But automated liquid culture systems have significant shortened turnaround times as compared with conventional solid media. These systems may not be feasible in laboratories in low to middle income countries with a high burden of TB and drug resistance TB due to lack of proper infrastructure, resources and trained personnel.[7,30]

The frequency and mutational patterns identified in our study are significantly different from a previous report.[27] We found that S531L mutation in the *rpoB* gene occurred very frequently (62.3%) among RIF resistant strains and the most frequent mutation found in MDR-TB strains. This is in accordance with finding from a recent South African study.[27] Other mutations were detected in the *rpoB* codon D516V in 8 (17.7%) and *rpoB* codon H526Y in 6 strains (11.1%). In these strains, the particular mutations could be clearly identified directly by hybridisation to specific oligonucleotide targeting the mutation sequence. For INH resistance, S315T1 mutation in the *katG gene* was most common 42 (93.3%) in our study. A previous study by van Rie *et al*. in 2001 reported that the mutation in the *katG* was less frequent (37.6%, $P = 0.01$).[31] Mutation in the 315 region of *katG* was present in 93.3% of all INH resistant isolates worldwide and predominantly reported from Germany, Russia and other countries.[14,27,32,33] High prevalence of *katG* mutation has been reported to confer resistance in high TB prevalence countries[29] due to presumably ongoing transmission of these strains.[14] In our study, frequency of mutation seen in INH-resistant strains was 28.9%; which carried a mutation in the *inhA* promoter region (invariably C15T), which was considerably lower than 40% reported in the Barnard's study ($P = 0.007$).[27]

A total of five (4%) MTBC clinical isolates were excluded from the study because of unreadable or having either no band at all or very light/weak/unreadable bands in *rpoB, katG* and/or *inhA* sections.[34] Although, only 45 MDR-TB strains have been evaluated with the GenoType® MTBDR*plus* assay in this study, but more number of MDR-TB strains may be required for strengthening epidemiological data of different mutations in this geographical region.

It is well known that DST of anti-tubercular drugs by conventional methods is difficult due to various technical reasons and the results are not always accurate.[35,36] In addition, it may take up to 6 weeks to get a phenotypic DST result and during this period the patient may infect his contacts. Recent studies have demonstrated the feasibility of the GenoType® MTBDR*plus* assay; which was utilised as an effective tool for MDR-TB screening in countries with a high burden of DR-TB and showed good concordance with phenotypic DST results.[24,27,28,37] However, rapid DST has a number of drawbacks, generally related to the low concentration of bacilli and possible presence of different types of Mycobacteria (i.e., mixture of sensitive and resistant clones) in the sputum specimen. The GenoType® MTBDR assay has short turnaround times in a high-volume facility to control and prevent new cases of MDR-TB in the society.

Conclusion

The GenoType® MTBDR*plus* assay is a highly sensitive, specific, reliable, rapid screening test for the detection and identification of different mutational patterns conferring resistance to MDR-TB cases. Our study demonstrated a high concordance of different resistance patterns observed between GenoType® MTBDR*plus* assay and conventional 1% proportion method by using the BacT/ALERT 3D system. It has a potential to substantially reduce the

turnaround times of conventional DST methods. It can be used even for smear positive clinical samples from suspected cases of treatment failure, recurrent DR-TB, and culture positive isolates from suspected DR-TB patients. This test may be a useful tool for WHO Global Task Force to control and prevent new cases of MDR-TB in the community.

Acknowledgment

This work was supported by grant from Indian Council of Medical Research, New Delhi (Extramural ICMR Project Sanction No. 5/8/5/4/2007-ECD-I). The authors would like to thank the Technical Member of Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, India, for their technical support during research work.

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How to cite this article: Maurya AK, Singh AK, Kant S, Umrao J, Kumar M, Kushwaha R, *et al.* Use of GenoType® MTBDR*plus* assay to assess drug resistance and mutation patterns of multidrug-resistant tuberculosis isolates in northern India. Indian J Med Microbiol 2013;31:230-6.

Source of Support: Indian Council of Medical Resaerch, New Delhi,
Conflict of Interest: None declared.