Profile of Drug-Resistant-Conferring Mutations among New and Previously Treated Pulmonary Tuberculosis Cases from Aligarh Region of Northern India

Shariq Ahmed¹, Indu Shukla¹, Nazish Fatima¹, Sumit K. Varshney¹, Mohammad Shameem², Uzma Tayyaba¹

Departments of ¹Microbiology and ²TB and Respiratory Diseases, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Abstract

Background: The prevalence of multidrug-resistant-tuberculosis (MDR-TB) among new and previously treated cases is increasing worldwide as well as in India. Rapid detection of MDR-TB allows the establishment of an effective treatment regimen; minimizes the risk of further resistance, and limits the spread of drug-resistant strains. Early diagnosis of MDR-TB is the need of the hour in high-TB burden countries like India, and GenotypeMTBDRplus is quite sensitive and specific in determining the molecular resistance in drugs such as rifampicin and isoniazid. Methods: The present study was done for molecular detection of rifampicin and isoniazid resistance and resistance patterns among MDR-TB suspects and comparison of resistance patterns among new and previously treated cases by GenoType® MTBDRplus Line Probe Assay. A total of 1268 sputum samples of MDR-TB suspects were subjected to fluorescent microscopy. Fluorescent microscopy positive samples were subjected to GenoType® MTBDRplus (HAIN Lifescience) assay. Results: MDR-TB was detected 11.02%, 20.03% in new and previously treated cases. Among MDR-TB patients S531 L was the most common mutation detected in rpoB gene; 71.43% in new, and 72.17% in previously treated cases. S315T1 was the most common mutation noted in katG gene; 100% in new and 81.74% in previously treated. While in aH gene, it was C15T (7.8%) among previously treated cases. Conclusion: MDR-TB has high prevalence in the western part of Uttar Pradesh, India. Previously treated cases have even more high rate of MDR-TB than new TB cases. The most dominant gene mutations associated with resistance to INH and RIF were observed in codon 315 of the katG gene and codon 531 of the rpoB gene. While comparing the mutation patterns by Genotype MTBDRplus assay, previously treated cases showed more diversity of mutations and had greater number of unknown mutations.

Keywords: inhA gene, katG gene, multi-drug resistant tuberculosis, rpoB gene

INTRODUCTION

The global threat of multidrug-resistant tuberculosis to control tuberculosis underscores the importance of prompt and rapid identification of such resistant Mycobacterium tuberculosis strains. Isoniaizid and rifampicin are the key first-line anti-tuberculosis drugs, and resistance to these drugs i.e., MDR-TB, is likely to result in treatment failure and poor clinical outcomes.⁴,⁵

Among patients with multidrug-resistant tuberculosis (MDR-TB), delays in diagnosis and treatment initiation are frequently observed, resulting in an increased risk of disease complications, high mortality, and pretreatment lost to follow-up rates. In addition, such delays lead to an extended period of TB infectivity within the community, resulting in higher transmission rates, higher mortality, and morbidity.⁴ The random mutation rate of $3 \times 10^{-7}$ per organism per generation is natural for first-line anti-tuberculosis drugs against TB that gives drug resistance.⁴⁵ This small proportion of naturally occurring drug-resistant mutants, however, rapidly multiplies due to activities such as inaccurate or incomplete chemotherapy. While resistance to INH is mainly associated with mutations in the katG, inhA, and ahpC genes, resistance

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Address for correspondence: Dr. Shariq Ahmed, Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh - 202 001, Uttar Pradesh, India. E-mail: shariqahmed0105@gmail.com

ORCID: Shariq Ahmed: https://orcid.org/0000-0002-4917-4015

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to RIF is predominantly linked to mutations in the \( rpoB \) gene.\(^{[4,7]} \)

The prevalence of MDR-TB among new and previously treated cases is increasing worldwide as well as in India.\(^{[8,9]} \)

Most of the burden of MDR-TB is mainly located in low- and middle-income countries.\(^{[10]} \)

The transmission of drug-resistant tuberculosis strains is increasing because of the growing burden of MDR-TB patients.\(^{[3,11]} \)

Rapid detection of MDR-TB allows the establishment of an effective treatment regimen; minimizes the risk of further resistance, and limits the spread of drug-resistant strains.\(^{[12]} \)

Due to the slow growth of \( M. \) \( tuberculosis \) bacilli, delays in the detection of resistant strains can occur when conventional phenotypic assays are used. Nucleic acid amplification-based techniques are potentially the most rapid and sensitive methods for detection, identification, and susceptibility testing and are theoretically able to provide same day diagnosis from clinical samples.\(^{[13-16]} \)

These methods can potentially reduce the diagnostic time from weeks to days.\(^{[17]} \)

The molecular basis of antitubercular drug resistance in \( M. \) \( tuberculosis \) is becoming clearer. More than 96% of rifampin-resistant strains have mutations in an 81-bp “core region” of the \( rpoB \) gene, which encodes the beta-subunit of the RNA polymerase\(^{[17,18]} \) and the majority of isoniazid-resistant strains have been found to contain mutations in codon 315 of the \( katG \) gene, which encodes the catalase-peroxidase\(^{[19]} \) or mutations in the \( inhA \) ribosomal binding site.\(^{[20]} \)

Line probe assay (LPA) genotype MTBDRplus assay is validated for both direct uses on smear-positive pulmonary specimens and on isolates of \( M. \) \( tuberculosis \) grown on liquid medium or in solid medium.\(^{[3]} \)

Genotype MTBDRplus assay is based on multiplex polymerase chain reaction combined with reverse hybridization on nitrocellulose strips targeting common mutations for RIF and INH resistance. The assay has an additional advantage over other LPA because the Genotype MTBDRplus assay identifies mutations in the \( rpoB \) gene (coding for the \( \beta \)-subunit of the RNA polymerase) for the detection of RIF resistance, mutations in the \( katG \) gene (coding for the catalase-peroxidase) for high-level INH resistance, and mutations in the promoter region of \( inhA \) gene (coding for the NADH enoyl-ACP reductase) for low-levels INH resistance.\(^{[4]} \)

A 2008 meta-analysis found that the GenoTypeMTBDRplus assay and another similar commercial test had a pooled sensitivity of 98% for detecting RIF resistance and 89% for detecting INH resistance and specificity of 99% for R and H.\(^{[21]} \)

In June 2008, the World Health Organization recommended the use of molecular LPA for the diagnosis of MDR-TB.\(^{[10]} \)

Above statements bring to our concern that early diagnosis of MDR-TB is the need of the hour in high TB burden countries like India, and GenotypeMTBDRplus is quite sensitive and specific in determining the molecular resistance in drugs such as rifampicin and isoniazid. Knowing the profile of mutations and their occurrence in new and previously treated cases of MDR-TB in a respective geographical location may help in better understanding of molecular diagnosis of MDR-TB. Therefore, this study was aimed to determine MDR-TB prevalence in new and previously treated cases, and the patterns of mutations in \( rpoB \) gene (for detecting RIF resistance), \( katG \) and \( inhA \) gene (for detecting INH resistance) in MDR-TB suspected patients with the help of Genotype MTBDRplus Line Probe Assay.

**METHODS**

**Ethical considerations**

The study was approved by the Institutional Ethics and Research advisory committee, Faculty of Medicine, Aligarh Muslim University, Aligarh.

**Study subjects**

The present study was conducted in Culture and DST Laboratory (RNTCP certified), Department of Microbiology on the sputum samples received of the MDR-TB suspected patients (according to PMDT guidelines) from the outpatient and in-patients departments of the hospital and from various tuberculosis units in and around Aligarh region from January 2015 to August 2016.

**Specimen collection and processing**

Sputum samples were collected as per RNTCP criteria (Central TB Division), which were subjected to fluorescent microscopy. The sputum specimens were handled in Class II biosafety cabinet and were decontaminated by N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method.\(^{[22,23]} \)

Subsequently, the sediments were suspended in 1–1.5 ml sterile phosphate buffer (pH 6.8) and two bottles of Lowenstein–Jensen medium were inoculated with each sample. 500 μl of the processed sample was used for DNA isolation in a screw-capped tube.

**Line probe assay**

The GenoType MTBDRplus LPA was performed according to the manufacturer’s (Hain Lifescience, Nehren, Germany) instructions.

**Interpretation**

Each strip of LPA had 27 reaction zones (bands) including six controls (conjugate, amplification, \( M. \) \( tuberculosis \) complex band (TUB), \( rpoB \), \( katG \) and \( inhA \) controls), eight \( rpoB \) wild-type (WT1–WT8) and four mutant probes (\( rpoB \) MUT DS16V, \( rpoB \) MUT HS26Y, \( rpoB \) MUT HS26D, and \( rpoB \) MUT S533 L), one \( katG \) wild-type and two mutant probes (\( katG \) MUT S315T1 and \( katG \) MUT 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. To give a valid result, all six expected control bands should appear correctly. Otherwise, the result is considered invalid.\(^{[23]} \)

**Statistical analysis**

Data were analyzed using http://www.socscistatistics.com/tests/ztest/Default2.aspx. Descriptive data were presented as
frequency (percentage). Z-score was used to calculate P value. P < 0.05 was considered statistically significant.

RESULTS
A total of 1268 sputum samples of MDR-TB suspected cases from a period of 18 months from January 2015 to August 2016 were included in the study. The samples received were classified as new TB cases and previously treated cases as per Revised National TB Control Programme guidelines.

Out of 1268 sputum samples from MDR-TB suspected patients, 194 (15.3%) were from new cases, and 1074 (84.7%) were from previously treated cases. All sputum samples were subjected to fluorescent microscopy of which 744 samples were positive. From the 744 fluorescent positive samples, 128 (17.2%) samples were from new cases, and 616 (82.8%) samples were from previously treated samples [Table 1].

Resistant pattern obtained online probe assay
All the 744 fluorescent positive samples were subjected to LPA. A total of 701 samples showed the presence of TUB band (M. tuberculosis complex) but was absent in 43 samples. Among the TUB band positive samples, 127 were new, and 574 were previously treated cases [Table 2].

Nearly 129 (18.40%) strains were found to be MDR that is, resistant to both drugs H as well as R and 459 (65.48%) were found sensitive for both drugs. However, monoresistance to isoniazid and rifampicin was detected in 69 (9.84%) and 44 (6.28%) strains, respectively, in both the cases [Table 3].

Resistance to isoniazid and rifampicin in new cases
In new cases, of the 127 strains 14 (11.02%) strains were MDR (R + H resistant), and 97 (76.37%) strains were sensitive to both drugs (R + H). 6 (4.72%) and 10 (7.87%) were monoresistant to rifampicin and isoniazid [Table 3].

Resistance to isoniazid and rifampicin in previously treated cases
In previously treated cases, of 574 strains, 115 (20.03%) strains were found MDR (R + H resistant), and 362 (63.07%) strains were sensitive to both drugs (R + H). Monoresistance to rifampicin and isoniazid were 38 (6.62%) and 59 (10.28%) [Table 3].

An analysis of the frequency and mutational patterns associated with MDR-TB as well as monoresistant strains

| Table 1: Distribution of pulmonary tuberculosis in New and previously treated cases (n=744) |
|----------------------------------|-----------------|-----------------|-----------------|
|                                  | New             | Previously treated | Total           |
|----------------------------------|-----------------|-----------------|-----------------|
| R + H resistant                  | 14 (11.02)      | 115 (20.03)     | 129 (18.40)     |
| Monoresistant R                   | 6 (4.72)        | 38 (6.62)       | 44 (6.28)       |
| Monoresistant H                   | 10 (7.87)       | 59 (10.28)      | 69 (9.84)       |
| R + H sensitive                  | 97 (76.37)      | 362 (63.07)     | 459 (65.48)     |
| Total                            | 127 (100)       | 574 (100)       | 701 (100)       |

**Table 2: The presence of TUB band among new and previously treated cases of Pulmonary Tuberculosis in fluorescent microscopy positive smear (n=744)**

|                                  | New cases (%) | Previously treated cases (%) | Total (%) |
|----------------------------------|---------------|-------------------------------|-----------|
| TUB band positive                | 127 (99.22)   | 574 (93.18)                  | 701 (94.22)|
| TUB band negative                | 1 (0.78)      | 42 (6.82)                    | 43 (5.73) |
| Total                            | 128 (100)     | 616 (100)                    | 744 (100) |

TUB: Mycobacterium tuberculosis complex

| Table 3: Antimycobacterial sensitivity profile of multidrug-resistant-Tuberculosis suspected patients as detected by line probe assay |
|---------------------------------------------------------------|-----------------|-----------------|-----------------|
|                                                              | New cases (%)   | Previously treated cases (%) | Total (%) |
| R + H resistant                                              | 14 (11.02)      | 115 (20.03)     | 129 (18.40)     |
| Monoresistant R                                               | 6 (4.72)        | 38 (6.62)       | 44 (6.28)       |
| Monoresistant H                                               | 10 (7.87)       | 59 (10.28)      | 69 (9.84)       |
| R + H sensitive                                              | 97 (76.37)      | 362 (63.07)     | 459 (65.48)     |
| Total                                                         | 127 (100)       | 574 (100)       | 701 (100)       |

R: Rifampicin, H: Isoniazid
were performed using the GenoType® MTBDRplus assay. Readable bands pattern of rpoB, katG, and inhA section in GenoType® MTBDRplus assay results were obtained from the 701 MTBC strains.

Table 4 shows the pattern of gene mutations detected by Genotype MTBDRplus assay in drug-resistant mycobacterium strains. In rpoB gene, band patterns among the 14 MDR (R + H) maximum no. of strains 7 (50.00%) showed the absence of WT-8 band corresponding to mutation in the gene region 530–533. None of the strains showed absence of WT2, WT4, WT5, WT6, WT7 band corresponding to gene region 510–513, 516–519, 518–522, 521–525, and 526–529. MUT 3 band appeared in most of the strains 10 (71.43%) corresponding to S531 L mutation. Similarly among the 50 monoresistant R strain rpoB gene band patterns again WT-8 band was absent in most of the strains 4 (66.67%) corresponding to mutation in the gene region 530-533. None of the strains showed the absence of any other band. MUT 3 band appeared in all of the strains 6 (100%) corresponding to S531 L mutation.

In the katG gene, among the 14 MDR strains absence of WT band was seen in 13 (92.86%) corresponding to gene region 315. Whereas the presence of MUT1 band was seen in 14 (100%) corresponding to the mutation in S315T. Similarly among 10 monoresistant isoniazid strains absence of WT band was seen in 5 (50%) corresponding to gene region 315. Whereas the presence of MUT1 band was 7 (70%) corresponding to mutation in S315T1 and MUT2 band corresponding to S315T2 was not seen in any sample. In the inhA gene, among the 14 MDR strains, no gene mutations were detected. However, among 10 monoresistant isoniazid absence of WT1 band was seen in 2 (20%) corresponding to gene region -15/-16 whereas the presence of MUT 1 band was seen in 2 (20%) corresponding to mutation in C15T.

S315T1 mutation in katG gene was more seen in MDR-TB strains (14/14) than monoresistant isoniazid strains (7/10) and was statistically significant with \( P \) value of 0.028. While the most common mutation (C15T) seen in inhA gene, among the new cases though seen more in monoresistant, INH strains were not statistically significant (\( P = 0.08 \)).

Table 5 shows the pattern of gene mutations detected by Genotype MTBDRplus assay in drug-resistant mycobacterium strains among previously treated cases. In rpoB gene, band patterns among the 115 MDR (R + H) maximum number of strains 73 (63.48%) showed the absence of WT-8 band corresponding to mutation in the gene region 530–533. WT 2, WT 3, WT 4, WT 5, WT 6, and WT 7 bands were absent in 5 (4.35%), 18 (15.65%), 13 (11.30%), 1 (0.87%), 1 (0.87%), 9 (7.83%) corresponding to mutation in the gene region 510–513, 513–517, 516–519, 518–522, 521–525, 526–529. None of the strains showed the absence of WT 1 band.

| Band | Gene region or mutation | MDR strains (R + H) \((n=14)\) (%) | RIF (R) mono-resistant \((n=6)\) (%) | INH (H) Mono-resistant \((n=10)\) (%) | \( P \) |
|------|-------------------------|------------------------------------|-------------------------------------|-------------------------------------|------|
| rpoB | WT1                     | 506–509                            | 1 (7.14)                            | 0                                   | 50   |
|      | WT2                     | 510–513                            | 0                                   | 0                                   |      |
|      | WT3                     | 513–517                            | 3 (21.43)                           | 0                                   | 22   |
|      | WT4                     | 516–519                            | 0                                   | 0                                   |      |
|      | WT5                     | 518–522                            | 0                                   | 0                                   |      |
|      | WT6                     | 521–525                            | 0                                   | 0                                   |      |
|      | WT7                     | 526–529                            | 0                                   | 0                                   |      |
|      | WT8                     | 530–533                            | 7 (50.00)                           | 4 (66.67)                           | 0.49 |
|      | MUT 1                   | D516V                               | 1 (7.14)                            | 0                                   | 50   |
|      | MUT 2A                  | H526Y                               | 0                                   | 0                                   |      |
|      | MUT 2B                  | H526D                               | 0                                   | 0                                   |      |
|      | MUT 3                   | S315L                               | 10 (71.43)                          | 6 (100)                             | 0.14 |
| katG | WT                      | 315                                 | 13 (92.86)                          | 5 (50.00)                           | 0.02 |
|      | MUT 1                   | S315T1                              | 14 (100)                            | 7 (70)                              | 0.03 |
|      | MUT 2                   | S315T2                              | 0                                   | 0                                   |      |
| inhA | WT1                     | −15/−16                             | 0                                   | 2 (20.00)                           | 0.08 |
|      | WT2                     | −8                                  | 0                                   | 0                                   |      |
|      | MUT 1                   | C15T                                | 0                                   | 2 (20.00)                           | 0.08 |
|      | MUT 2                   | A16G                                | 0                                   | 0                                   |      |
|      | MUT 3A                  | T8C                                 | 0                                   | 0                                   |      |
|      | MUT 3B                  | T8A                                 | 0                                   | 0                                   |      |

R: Rifampicin, H: Isoniazid, INH: Isoniazid, MDR: Multi drug resistant, RIF: Rifampicin resistance
corresponding to gene region 506–509. MUT 3 band appeared in most of the strains 83 (72.17%) corresponding to S531 L mutation. Similarly, among the 38 monoresistant R strain \( \text{rpoB} \) gene band patterns again WT-8 band 19 (50%) was absent in most of the strains corresponding to mutation in the gene region 530–533. None of the strains showed the absence of WT 6 band 0 (0%) corresponding to gene region 521–525 and WT-1 (0%) corresponding to gene region 506–509. MUT 3 band appeared in most of the strains 22 (57.89%) corresponding to S531 L mutation. MUT 1, MUT 2A, and MUT 2B was seen in 2 (5.26%), 5 (13.16%), 2 (5.26%) strains corresponding to D516V, H526Y, and H526D mutations.

In the \( \text{katG} \) gene, among the 115 MDR strains, the absence of WT band was seen in 99 (86.09%) corresponding to gene region 315 whereas the presence of MUT1 band was seen in 94 (81.74%) and 2 (1.74%) corresponding to mutation in S315T1 and S315T2. MUT 1 and MUT 3A bands was seen in 9 (15.25%) and 1 (1.70%) corresponding to mutation in C15T and T8C.

Among the previously treated cases absence of WT 2 band corresponding to the mutation in region 510–513 of \( \text{rpoB} \) association with rifampicin monoresistant strains as compared to MDR-TB strains was statistically significant \((P = 0.017)\). While the association of mutation in katg gene (315 region) with MDR-TB strains as compared to INH monoresistant strains was also statistically significant \((P = 0.03)\).

Table 6 shows the pattern of gene mutations detected by genotype MTBDRplus assay in drug-resistant mycobacterium strains. In \( \text{rpoB} \) gene, band patterns among the 129 MDR \((R + H)\) maximum number of strains 80 (62.02%) showed the absence of WT 8 band corresponding to mutation in the gene region 530–533, next to it was absence of WT 3, WT 4, WT 7, WT 2 band in 21 (16.38%), 13 (10.08%), 9 (6.98%), and 5 (3.88%), respectively. The absence of WT 1, WT 5, WT 6 band were in 1 strain each only. MUT 3 band appeared in most of the strains 121 (67.22%) corresponding

| Band | Gene region or mutation | MDR strains \((R + H)\) \((n=115)\) (%) | RIF (R) monoresistant \((n=38)\) (%) | INH (H) Monoresistant \((n=59)\) (%) | \(P\) |
|------|--------------------------|-----------------------------|-----------------------------|-----------------------------|------|
| \( \text{rpoB} \) | WT1 | 506-509 | 0 | 0 | - |
| | WT2 | 510-513 | 5 (4.35) | 6 (15.79) | 0.02 |
| | WT3 | 513-517 | 18 (15.65) | 6 (15.79) | 0.98 |
| | WT4 | 516-519 | 13 (11.30) | 5 (13.16) | 0.76 |
| | WT5 | 518-522 | 1 (0.87) | 1 (2.63) | 0.41 |
| | WT6 | 521-525 | 1 (0.87) | 0 | 0.56 |
| | WT7 | 526-529 | 9 (7.83) | 2 (5.26) | 0.60 |
| | WT8 | 530-533 | 73 (63.48) | 19 (50) | 0.14 |
| | MUT 1 | D516V | 6 (5.22) | 2 (5.26) | 0.99 |
| | MUT 2A | H526Y | 6 (5.22) | 5 (13.16) | 0.10 |
| | MUT 2B | H526D | 4 (3.48) | 2 (5.26) | 0.62 |
| | MUT 3 | S531L | 83 (72.17) | 22 (57.89) | 0.10 |
| \( \text{katG} \) | WT | 315 | 99 (86.09) | 43 (72.89) | 0.03 |
| | MUT 1 | S315T1 | 94 (81.74) | 42 (71.17) | 0.11 |
| | MUT 2 | S315T2 | 2 (1.74) | 0 | 0.30 |
| \( \text{inhA} \) | WT | −15/−16 | 11 (9.57) | 10 (16.95) | 0.15 |
| | WT2 | −8 | 3 (2.61) | 1 (1.70) | 0.70 |
| | MUT 1 | C15T | 9 (7.83) | 9 (15.25) | 0.13 |
| | MUT 2 | A16G | 1 (0.87) | 0 | 0.48 |
| | MUT 3A | T8C | 0 | 1 (1.70) | 0.16 |
| | MUT 3B | T8A | 0 | 0 | - |

R: Rifampicin, H: Isoniazid, INH: Isoniazid, MDR: Multi drug resistant, RIF: Rifampicin resistance
to S531 L mutation. Similarly among the 44 monoresistant R strain $rpoB$ gene band patterns again WT 8 band was absent in most of the strains 23 (52.27%) corresponding to mutation in the gene region 530–533. None of the strains showed the absence of WT 1, WT 6 band corresponding to gene region 506–509 and 521–525. MUT 3 band appeared in most of the strains 28 (63.64%) corresponding to S531 L mutation.

In the $katG$ gene, among the 129 MDR strains absence of WT band was seen in 112 (86.82%) corresponding to gene region 315. Whereas the presence of MUT1 and MUT2 band was seen in 108 (83.72%) and 2 (1.55%) corresponding to mutation in S315T1 and S315T2. Similarly, among 69 monoresistant isoniazid strains the absence of WT band was seen in 48 (69.57%) corresponding to gene region 315. Whereas the presence of MUT1 band was 49 (71.01%) corresponding to mutation in S315T1. None of the strains shows the absence of MUT2 band.

In the $inhA$ gene, among the 129 MDR strains absence of WT band was seen in 112 (86.82%) corresponding to gene region 315. Whereas the presence of MUT1 and MUT2 band was seen in 108 (83.72%) and 2 (1.55%) corresponding to mutation in S315T1 and S315T2. Similarly, among 69 monoresistant isoniazid strains the absence of WT band was seen in 48 (69.57%) corresponding to gene region 315. Whereas the presence of MUT1 band was 49 (71.01%) corresponding to mutation in S315T1. None of the strains shows the absence of MUT2 band.

Table 6 shows among all the rifampicin-resistant strains detected by mutation in $rpoB$ gene by Genotype MTBDRplus it was seen that most common absence of simultaneous WT 3, WT 4 was seen in new 2 (10%), previously treated 8 (5.23%) and 10 (5.78%) total cases. Corresponding

| Band | Gene region or mutation | MDR strains (R + H) $(n=129)$ (%) | RIF (R) mono-resistant $(n=44)$ (%) | INH (H) mono-resistant $(n=69)$ (%) | P |
|------|-------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---|
| $rpoB$ | WT1 | 506–509 | 1 (0.78) | 0 | 0.56 |
| WT2 | 510–513 | 5 (3.88) | 6 (13.64) | 0.02 |
| WT3 | 513–517 | 21 (16.38) | 6 (13.64) | 0.67 |
| WT4 | 516–519 | 13 (10.08) | 5 (11.36) | 0.81 |
| WT5 | 518–522 | 1 (0.78) | 1 (2.27) | 0.42 |
| WT6 | 521–525 | 1 (0.78) | 0 | 0.56 |
| WT7 | 526–529 | 23 (62.02) | 80 (62.02) | 0.25 |
| WT8 | 530–533 | 6 (13.64) | 6 (13.64) | 0.81 |
| MUT 1 | D516V | 5 (3.88) | 5 (11.36) | 0.11 |
| MUT 2A | H526Y | 1 (0.78) | 1 (2.27) | 0.42 |
| MUT 2B | H526D | 1 (0.78) | 1 (2.27) | 0.42 |
| MUT 3 | S531L | 93 (72.10) | 28 (63.64) | 0.29 |
| $katG$ | WT | 315 | 112 (86.82) | 48 (69.57) | 0.003 |
| MUT 1 | S315T1 | 108 (83.72) | 49 (71.01) | 0.04 |
| MUT 2 | S315T2 | 2 (1.55) | 0 | 0.30 |
| $inhA$ | WT1 | –15/−16 | 11 (8.53) | 12 (17.39) | 0.30 |
| WT2 | –8 | 3 (2.33) | 1 (1.45) | 0.67 |
| MUT 1 | C15T | 3 (2.33) | 1 (1.45) | 0.67 |
| MUT 2 | A16G | 2 (1.55) | 1 (1.45) | 0.17 |
| MUT 3A | T8C | 0 | 0 | - |
| MUT 3B | T8A | 0 | 0 | - |

R: Rifampicin, H: Isoniazid, INH: Isoniazid, MDR: Multi drug-resistant, RIF: Rifampicin resistance

As shown in Table 7, among all the 173 rifampicin-resistant isolates detected by Genotype MTBDRplus assay due to mutation in $rpoB$ gene, those samples that had absence of only one wild-type band or presence of one MUT band; S531 L mutation was the most common mutation seen in both new 16 (80.0%) and previously treated cases 100 (65.36%). H526Y was the second most common mutation detected in previously treated cases 7 (4.58%) while in new cases in was D516V 1 (5.00%).

Similarly, in all the 198 isoniazid-resistant isolates detected by mutation in $katG$ gene; S315T1 was the most common mutation detected in new 21 (87.5%), previously treated 136 (78.16%) as well as total cases 157 (79.29%). Isoniazid-resistant isolates detected by mutation in $inhA$ gene C15T was the most common mutation detected in new 2 (8.33%), previously treated 17 (9.77%), and total cases 19 (9.60%) [Table 7].

Table 8 shows among all the rifampicin-resistant strains detected by mutation in $rpoB$ gene by Genotype MTBDR plus it was seen that most common absence of simultaneous WT 3, WT 4 was seen in new 2 (10%), previously treated 8 (5.23%), and 10 (5.78%) total cases. Corresponding
mutations seen were the absence of WT7 (region 526–529) and presence of MUT2A band i.e., H526Y mutation in new 0 (0%), previously treated 7 (4.58%) and total cases 7 (4.05%). Similarly, WT7, MUT2B (H526D) were 0 (0%), 4 (2.61%), 4 (2.31%); WT8, MUT2A (H526Y) were 0 (0%), 1 (0.65%), 1 (0.57%); WT8, MUT3 (S531L) were 16 (80.0%), 100 (65.36%), 116 (67.05%); WT3, WT4, MUT1 (D516V) were 1 (5%), 6 (3.92%), 7 (4.05%) in new, previously treated and total cases.

**Table 7: Single mutations observed using Genotype MTBDR-plus assay**

| LPA probes | Mutation site | Mutation detected | Number of isolates |
|------------|---------------|-------------------|--------------------|
| **rpoB**   |               |                   |                    |
| WT 1       | 506-509       | UK                | 1 (5.00)           |
| WT 2       | 510-513       | UK                | 0                  |
| WT 3       | 513-517       | UK                | 2 (1.31)           |
| WT 4       | 516-519       | UK                | 0                  |
| WT 5       | 518-522       | UK                | 0                  |
| WT6        | 521-525       | UK                | 0                  |
| WT 7       | 526-529       | UK                | 2 (1.31)           |
| WT8        | 530-533       | UK                | 0                  |
| MUT 1      | 516           | D516V             | 1 (5.00)           |
| MUT 2A     | 526           | H526Y             | 7 (4.58)           |
| MUT 2B     | 526           | H526D             | 4 (2.61)           |
| MUT 3      | 531           | S531L             | 16 (80.0)          |
| **inhA**   |               |                   |                    |
| WT 1       | −15/−16       | UK                | 0                  |
| WT2        | −8            | UK                | 0                  |
| MUT 1      | −15           | C15T              | 2 (8.33)           |
| MUT 2      | −16           | A16G              | 0                  |
| MUT 3A     | −8            | T8C               | 0                  |
| MUT 3B     | −8            | T8A               | 0                  |

R: Rifampicin, INH: Isoniazid, MDR: Multidrug resistant, LPA: Line probe assay

**Discussion**

In the present study, 1268 sputum samples of MDR-TB suspected patients were subjected to microscopy. 194 (15.3%) were from new cases, and 1074 (84.7%) were from previously treated cases. Out of these, 744 light-emitting diode-fluorescent microscopy positive samples were tested by LPA. Genotype MTBDRplus LPA from Nehren Germany was used to get the result. A total of 701 samples showed the presence of TUB band (M. tuberculosis complex). It was absent in 43 samples. It was seen that most of the strains that were TUB band negative 42 (6.82%) belong to previously treated cases. These TUB band negative samples were previously treated, and 2 (1.01%) total cases. Corresponding mutation seen by the absence of WT1, WT2 (−15/−16,−8) presence of MUT1 (C15T) were 0 (0%), 1 (0.57%), 1 (0.52%); WT1, MUT1 (C15T) were 2 (8.33%), 16 (9.20%), 18 (9.38%); WT2, MUT3A (T8C) were 0 (0%), 1 (0.57%), 1 (0.52%) in new, previously treated and total cases.
found to be non-TB Mycobacterium on the basis of their growth characteristics, pigmentation, and by biochemical identification. Thakur et al.\[24\] also reported 521 (90.76\%) as MTBC positive and 53 (9.23\%) as MTBC negative similar to our findings.

Multidrug resistance can be conferred by a single mutational event the genes coding for multidrug efflux transporters, membrane proteins that recognize dissimilar toxic compounds and pump them out of eukaryotic and bacterial cells. Although efflux pump genes have been identified in mycobacteria, and M. tuberculosis, in particular, they do not seem to play a major role in the emergence of MDR strains.\[25‑28\] To the contrary, multi-drug resistance is thought to be the consequence of stepwise accumulation of random mutations in the chromosome selected under the environmental pressure of chemotherapy. These findings have important implications for treatment program strategy.\[28,29\] Drug resistance in M. tuberculosis occurs by random, single step, spontaneous mutation at a low but predictable frequency, in large bacterial populations. The probability of incidence of drug-resistant mutants is $10^{-8}$ for rifampicin, while for isoniazid and some of the other commonly used drugs it is $10^{-6}$. Therefore, the probability for resistance to both isoniazid and rifampicin to develop is $10^{-14}$, which is much larger than the number of organisms present in a medium-sized cavity in a patient with open pulmonary TB.\[30\]

In our study, 129 (18.40\%) strains were found to be MDR that is, resistant to both drugs isoniazid as well as rifampicin. Monoresistance to isoniazid and rifampicin were detected in 69 (9.84\%) and 44 (6.28\%) strains, respectively. Other studies from across India depicted MDR, monoresistant RIF and monoresistant INH as 29.41\%, 2.94\% and 8.82\%; 42\%, 11\%, and 10\%; 55.5\%, 14.8\%, and 9.2\%; 19.76\%, 6.14\%, and 8.63\%; 28\%, 1\%, and 10\%; respectively. Maurya et al.\[31\] reported 13.4% MDR‑TB in extrapulmonary tuberculosis cases.

MDR‑TB suspected patients were divided into new cases and previously treated cases. Resistance to both rifampicin and isoniazid were seen in 14 (11.02\%) new cases. Whereas in previously treated cases, it was 115 (20.03\%). Monoresistance to rifampicin and isoniazid in new cases were seen in 6 (4.72\%) and 10 (7.87\%) patients. Similarly, monoresistance to rifampicin and isoniazid in previously treated cases were seen in 38 (6.62\%) and 59 (10.28\%).

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**Table 8: Multiple/corresponding mutation patterns observed using Genotype MTBDR-plus assay**

| LPA probes | Mutation site | Mutation detected | Number of isolates |
|------------|---------------|--------------------|--------------------|
|            |               |                    | New cases (n=20) (%) | Previously treated cases (n=153) (%) | Total (new + previous) (n=173) (%) |
| **rpoB**   |               |                    |                     |                           |                                |
| WT 2, WT 3 | 510‑517       | UK                 | 0                   | 3 (1.96)                  | 3 (1.73)                       |
| WT2, WT3, WT4 | 510‑519     | UK                 | 0                   | 3 (1.96)                  | 3 (1.73)                       |
| WT 3, WT 4 | 513‑519       | UK                 | 2 (10)              | 8 (5.23)                  | 10 (5.78)                      |
| WT 4, WT 5 | 516‑522       | UK                 | 0                   | 1 (0.65)                  | 1 (0.57)                       |
| WT 3, WT 7 | 513‑517, 526‑529 | UK               | 0                   | 2 (1.31)                  | 2 (1.16)                       |
| WT7, MUT 2A | 526‑529     | H526Y              | 0                   | 1 (0.65)                  | 1 (0.57)                       |
| WT 7, MUT 2B | 526‑529     | H526D              | 0                   | 3 (1.96)                  | 3 (1.73)                       |
| WT8, MUT1, MUT2A | 530‑533  | D516V              | 0                   | 1 (0.65)                  | 1 (0.57)                       |
| WT8, MUT 2A | 530‑533      | H526Y              | 0                   | 1 (0.65)                  | 1 (0.57)                       |
| WT8, MUT 3 | 530‑533      | S531L              | 11 (55)             | 90 (58.82)                | 101 (58.38)                     |
| WT3, WT4, MUT1 | 513‑519   | D516V              | 1 (5)               | 6 (3.92)                  | 7 (4.05)                       |

| **LPA probes** | Mutation site | Mutation detected | Number of isolates |
|----------------|---------------|--------------------|--------------------|
|               |               |                    | New cases (n=24) (%) | Previously treated cases (n=174) (%) | Total (new + previous) (n=198) (%) |
| **katG**      |               |                    |                     |                           |                                |
| WT, MUT1      | 315           | S315T1             | 17 (70.83)          | 125 (71.83)               | 142 (73.93)                     |
| WT, MUT2      | 315           | S315T2             | 0                   | 2 (1.15)                  | 2 (1.01)                       |
| **inhA**      |               |                    |                     |                           |                                |
| WT 1, WT 2   | −15/‑16, −8   | UK                 | 0                   | 2 (1.15)                  | 2 (1.01)                       |
| WT1, WT2, MUT1 | −15/‑16, −8  | C15T               | 0                   | 1 (0.57)                  | 1 (0.52)                       |
| WT1, MUT1    | −15/‑16       | C15T               | 2 (8.33)            | 16 (9.20)                 | 18 (9.38)                      |
| WT2, MUT3A   | −8            | T8C                | 0                   | 1 (0.57)                  | 1 (0.52)                       |

R: Rifampicin, INH: Isoniazid, MDR: Multidrug-resistant, LPA: Line probe assay
Primary drug resistance to isoniazid in the present study was found to be 18.89% which was quite similar to other reported studies in India such as New Delhi 18.5%,[18] Jabalpur 17%, Raichur 18.7%,[19] higher than Tamil Nadu 15.4%, Wardha 15%,[20] Jaipur 13.6%,[21] and Ranchi 2.86%;[22] lower than Himachal Pradesh[23] that showed 21.35% resistance in newly diagnosed cases. However, primary drug resistance to isoniazid in other countries was reported from Uganda 5.8%, Germany 7.1%, and Australia 8.9%.[24] In other studies, higher resistance was reported from Bangladesh with 54.5%[25] and Saudi Arabia with 33.8%.[26]

On the other hand, primary drug resistance to rifampicin in the present study was observed to be 15.74% which is lower to the study done by Thakur et al. 2015[27] that showed 19.42% resistance but was higher as compared to other studies in India; New Delhi 0.6%,[28] Wardha 0.5%, Jabalpur 2% and Raichur 2.5%,[29] Bengaluru 2.6%,[30] Ranchi 3.51%,[31] Tamil Nadu 4.4%,[32] Lucknow 4.7%,[33] and Jaipur 6.8%.[34] Internationally, resistance to rifampicin was reported from China 6.6%, Uganda 1.5%, Germany 1.6%, and Australia 2.6%.[35] Higher resistance was reported from Saudi Arabia 23.5%[36] and Bangladesh 50%. [37]

Overall MDR-TB cases gradually decreased from 2009 to 2015 in both new and previously treated cases in Northern India, but still the prevalence rate is higher in North India than National Prevalence rate of MDR-TB.[38] The rate of primary MDR-TB in our study was 11.02% that was slightly lower to that reported from Himachal 12.62%[39] that is comparatively higher to several reports elsewhere in India, Lucknow 4.7%[40] and Jaipur 4.5%.[41] A study from Mumbai revealed the highest proportion of MDR-TB in new cases with 24%.[42] MDR-TB rate was also reported from other countries such as China 5.7%,[43] Bangladesh 40.9%,[44] Saudi Arabia 20.6%,[45] and Philippines 2%.[46] In our study, higher rate of primary drug resistance was observed as compared to other studies in India, as patients included in this study were exclusively MDR-TB suspects.

Acquired drug resistance to isoniazid in the present study was found to be 30.31%, which was similar to that in Himachal Pradesh with 30.14%,[47] lower than Mumbai with 53.2%,[48] Jaipur 39.7%[49] from Indian studies. This is also lower than Ethiopia with 56.1%, Bangladesh 82.6%, and China 38.8%, but higher than Germany where it is 15.4%, Sri Lanka 5.3%, and Uganda 20% in addition to the very similar rate of resistance reported from Australia 29.2% from the studies of other countries.[49]

Similarly, acquired drug resistance to rifampicin in the present study was 26.65%, a very similar finding reported from Jaipur 28.2%[50] and Himachal Pradesh 27.51%.[51] Higher rates of resistance were reported by other studies in India from Mumbai with 74.4%[52] and New Delhi 33.7%.[53] It is also higher in other countries such as Ethiopia with 46.1%[54] China 29.7%,[55] Uzbekistan 62.5%[56] and Bangladesh 80%.[40] however, lower rate of resistance reported from Uganda with 13.4%, Germany 7.7%, and Sri Lanka 2.6%.[57] Acquired MDR-TB in the present study was 20.03%. Similar resistance was reported from Jaipur 24.3%[58] and Himachal Pradesh with 21.53%[59] but higher than Bengaluru 12.8%.[60] Comparatively higher resistance was reported at New Delhi with 33.79%[61] and Mumbai 41%.[62] Studies from Philippines[63] and Nigeria[64] reported 21% and 3.1%.

Collectively, DNA sequencing studies demonstrate that more than 95% of RIF-resistant M. tuberculosis strains have a mutation within the 81-bp hotspot region of the rpoB gene.[18,52,53] An analysis of the frequency and mutational patterns associated with MDR-TB as well as monoresistant strains was performed using the GenoType MTBDRplus assay. Readable bands pattern of rpoB, katG, and inhA section in GenoType MTBDRplus assay results were obtained from the 701 MTBC strains (127 from new cases and 574 from previously treated cases). Among the 173 rifampicin resistant and 198 isoniazid-resistant strains, 147 (83.04%), 159 (80.30%), and 22 (11.11%) strains harbored known mutation in rpoB, katG, and inhA genes, respectively. Similar studies from India and other countries having high prevalence of TB reported mutations in rpoB, katG, and inhA genes, respectively. Similar studies from India and other countries having high prevalence of TB reported mutations in rpoB, katG, and inhA genes, respectively. Similar studies from India and other countries having high prevalence of TB reported mutations in rpoB, katG, and inhA genes, respectively. Similar studies from India and other countries having high prevalence of TB reported mutations in rpoB, katG, and inhA genes, respectively.

Van Rie et al.[65] reported in 2001 that rifampicin resistance was highly associated with mutation in the 81 base pair region of the rpoB gene. Rifampicin resistance is known to be associated with mutations in 81 base pair region (codon 527–533) of the rpoB gene.[52,53,66] In this study, we also found that the most common mutation is associated with rpoB 530-533 region, mostly S531 L mutation (69.94%). This mutation was more frequently found in MDR-TB strains (72.10%) than in rifampicin monoresistant strains (63.64%); however, it was not statistically significant (P = 0.29). Similar findings were reported in Himachal Pradesh by Thakur et al.[67] which showed 62.2% mutation in S531 L region among all rifampicin resistant and 64.07% among MDR and 56.25% among rifampicin monoresistant. Other studies conducted in India; from Karnataka (72.2%),[68] New Delhi (59.76%),[69] Uttar Pradesh (62.3%),[70] New Delhi (47%),[71] and New Delhi (72%)[12] also showed similar results. Outside India, it was seen that similar results were detected by Albert et al.[72] in Uganda (47.61%), Hillemann et al. 2007[73] from Germany (73.6%), Sharma et al.[74] from Nepal (50%), and Cavusoglu et al. 2006[75] from Turkey (56.1%). Most of the above-mentioned studies also showed that there was no significant difference in the prevalence of this S531 L mutation in MDR-TB specimen and rifampicin monoresistance specimen.

Other mutations were detected in the rpoB codon such as D516V (5.20%), H526Y (6.36%) and H526D (3.47%). Thakur et al.[24] detected in the rpoB codon, such as D516V (4.44%),
H526Y (5.18%) and H526D (2.96%) which were quite similar to our results. 506-509, 521-525 region in the rpoB gene showed only one mutation in the 173 rifampicin-resistant samples in our study. Raizada et al.[61] showed 3.75% mutation in this region.

In both new and previously treated cases, S531 L mutation was the most common mutation detected. However, it was higher in new 16 (80%) than in previously treated 105 (68.63%). However, it was not statistically significant (P = 0.30).

The molecular basis of resistance to INH is more complex and involves mutations in more than one gene or gene complex such as the katG, inhA, and kasA genes and the intergenic region of the oxyR-ahpC complex.[61, 62] In Genotype MTBDR plus, INH resistance is detected by probes of two genes; katG and inhA. Van Rie et al.[59] reported that the mutation in the katG was less frequent (37.6%). Studies from a number of countries have been reported variability in the association of isoniazid resistance with mutations in katG or inhA.[63] Mutation in the 315 region of katG was 93.3%, present in all INH-resistant isolates worldwide and predominantly reported from Germany, Russia, and other countries.[6, 23, 64] High level and low level of INH resistance were shown to be associated with codon 315 of katG gene (50%–90%) and regulatory region of inhA gene (20%–35%), respectively, by various studies.[45, 65, 66]

The most frequent mutation found in INH-resistant strains was katG mutations 181/198 (91.41%) which occurred more commonly in MDR-TB strains (118/129 [91.47%]) compared to INH monoresistant strains (56/69 [81.16%]). Overall the frequency of inhA mutation was 26/198 (13.13%) and was lower in MDR-TB strains (11/129 [8.53%]) as compared to INH monoresistant strains (14/69 [20.29%]). Both the findings were statistically significant P value for the former and latter being 0.03 and 0.02, respectively. Very similar findings were reported from AIIMS New Delhi[23] and Karnataka.[66]

The mutations causing INH resistance are located in several regions.[66] Approximately, 34.6–94.3% of INH-resistant strains have been found to contain mutations in codon 315 of the katG gene.[6, 67–70] 2.9%–21.5% contain mutations in the inhA promoter region.[6, 68, 70] and an additional 2%–11.5% have mutations in the ahpC-oxrR intergenic region.[6, 68, 70]

Isoniazid resistance was most commonly associated with katG S315T1 mutation, in many high-TB burden countries, presumably related to ongoing transmission of these strains.[58, 70] In the present investigation, 80.81% mutation in the codon S315T1 that led amino acid serine substitution to threonine among the all INH-resistant strains, it was 83.72% in MDR strains and 71.01% in INH monoresistant strains. S315T2 mutation was less common (1.55%). In this study, the confirmed mutation in inhA gene was found (10.10%) in the promoter region that led amino acid substitution of cysteine to threonine in C15T region and 1 (1.45%) in T8C region.

These findings of the present study are within the range of various studies conducted all over India like Thakur et al.[24] showed that 74.32% mutation in the codon S315T1 (katG) and 5.40% in the C15T (inhA) region, Yadav et al.[23] S315T1 (83.91%), C15T (17.24%), Singhal et al.[53] reported INH-resistant isolates, 73/86 (84.88%) of which 11 (12.79%) had mutation in codon C15T and 1 (1.16%) in T8C. Maurya et al.[54] also showed that the most prominent mutations in katG and inhA genes were in S315T1 (93.3%) and 8 in C15T (17.7%). Raizada et al.[32] reported S315T1 (71.33%) and C15T (7.64%). Ranganath et al.[62] also showed S315T1 in 84.46%, and 14.70% in C15T and 2.94% in T8C. From worldwide, Tadesse M et al.[73] Albert and et al. 2010,[58] Tukvadze et al.,[71] Barnard et al.,[64] and Kiepiela et al.[72] also reported similar findings.

Among various mutations observed using Genotype MTBDRplus assay, majority of the drug-resistant isolates had multiple mutations (21/173 i.e., 12.13% for RIF resistance, 3/198 i.e., 1.52% for INH resistance) in our study. Multiple mutations were more common in previously treated cases (12.41%) than new cases (10%) in all rifampicin-resistant isolates. These multiple mutations are believed to be more probable in high-TB incidence places. However, the P value came out to be 0.75 which was not statistically significant.

Although most common single mutation in rpoB gene among new and previously treated cases was S531 L, it was higher in the former (80%) than in the latter (65.36%). Previously treated cases had strains having other mutations such as H526Y (4.45%) and H526D (2.61%) which were not seen in new cases strains. Similarly, in katG gene, most common mutation was S315T1 among new (87.5%) and previously treated (78.16%) cases. S315T2 mutation was seen only in previously treated cases.

Previously treated cases showed more diversity in mutations in all three genes. Like in rpoB gene absence of WT2, WT3, and WT7, bands were seen in previously treated cases but no corresponding MUT band was present implying that mutations would have occurred other than the common mutations (D516V, H526Y, H526D, and S531 L) which are detected by GenotypeMTBDRplus assay.

17 strains from previously treated showed only the absence of wild-type band corresponding to 315 region in the katG gene but no MUT band appeared which implies that mutation was not S315T1 or S315T2 rather some other mutations had occurred. Similarly, in inhA, among the previously treated cases, two strains showed the absence of WT1 band but no corresponding MUT band implying mutations C15T, A16G detected by genotype MTBDRplus did not occur in the 15/-16 region but some other mutation have occurred. As depicted in our other study[73] when 72 random samples were compared to conventional phenotypic DST, two samples which were detected sensitive for isoniazid with LPA were resistance on solid DST, the reason may be that presumably, these strains carry a mutation in other genomic regions not analyzed in this investigation, such as ahpC, kasA, iniA, iniB, iniC, efpA, furA, or ndh.[54, 70, 74]
CONCLUSION

From these observations, we can comment that MDR-TB has high prevalence in the western part of Uttar Pradesh, India. Previously treated cases have even more high rate of MDR-TB than new TB cases. The most dominant gene mutations associated with resistance to INH and RIF were observed in codon 315 of the katG gene and codon 531 of the rpoB gene. While comparing the mutation patterns by Genotype MTBDRplus assay, previously treated cases showed more diversity of mutations and had a greater number of unknown mutations that are not included in the strips. Phenotypic and genotypic approaches are therefore complimentary for obtaining high sensitivity and specificity in detecting drug resistance and susceptibilities to accurately predict MDR-TB cure and to gather the relevant data for resistance surveillance. More pooled data is required from across India to provide a standardized and comprehensive approach for the interpretation of mutations as predictors of MDR-TB drug-resistant phenotype.

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Conflicts of interest

There are no conflicts of interest.

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