Detection of multi-drug resistance & characterization of mutations in *Mycobacterium tuberculosis* isolates from North-Eastern States of India using GenoType MTBDRplus assay

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**Background & objectives:** Information on drug resistance tuberculosis is sparse from North-East (N-E) States of India. We undertook this study to detect multi-drug resistant tuberculosis (MDR-TB) among MDR-TB suspects, and common mutations among MDR-TB cases using GenoType MTBDRplus.

**Methods:** All MDR suspect patients deposited sputum samples to peripheral designated microscopy centres (DMC) in North-East States. The district TB officers (DTOs) facilitated the transport of samples collected during January 2012 to August 2012 to our laboratory. The line probe assay to detect common mutations in the *rpoB* gene for rifampicin (RIF) and *katG* and *inhA* genes for isoniazid (INH), respectively was performed on 339 samples or cultures.

**Results:** A total of 553 sputum samples from MDR suspects were received of which, 181 (32.7%) isolates were found to be multi-drug resistant. Missing WT8 along with mutation in codon S531L was commonest pattern for rifampicin resistant isolates (65.1%) and missing WT along with mutations in codon S315T1 of *katG* gene was commonest pattern for isoniazid resistant isolates (86.2%). Average turn-around time for dispatch of LPA result to these States from cultures and samples was 23.4 and 5.2 days, respectively.

**Interpretations & conclusions:** The MDR-TB among MDR-TB suspects in North-Eastern States of India was found to be 32.7 per cent. The common mutations obtained for RIF and INH in the region were mostly similar to those reported earlier.

**Key words** Drug-resistant - isoniazid - line probe - line probe assay - MDR-TB - rifampicin - tuberculosis

Multi-drug resistant tuberculosis (MDR-TB) poses grave challenge because of prolonged, limited and expensive treatment options with 10 to 30 per cent of cases resulting in failure of treatment and death¹. Timely diagnosis and prompt treatment of infectious cases is crucial in curtailing the spread of infection in the community. Conventional drug susceptibility (DST) has been the gold standard but takes upto 4-6 wk after the growth of bacteria. Liquid culture methods, are sensitive and faster but involve prohibitive expenditure². Commercial line probe assays (LPAs) based on reverse hybridization of amplicons to immobilized membrane...
based probes covering wild type and mutation sequences have been developed for rapid detection of MDR-TB. Results of GenoType MTBDRplus for rifampicin (RIF) and isoniazid (INH) resistance are comparable to conventional DST. Rifampicin resistance is caused by a mis-sense mutation in the beta-subunit of DNA dependent RNA polymerase in 81-bp hotspot region of rpoB gene. Resistance to INH is most frequently associated with mutations in kat G gene and inhA gene.

The Programmatic Management of Drug Resistant Tuberculosis (PMDT) guidelines in India, identify MDR-TB in suspects using GenoType MTBDRplus (WHO approved) only where it is available. The National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India is a referral TB hospital and National Reference Laboratory (NRL) for North-Eastern States of India. There are barely any data on MDR-TB from North-East (NE) India. Therefore, this study was aimed at detection of MDR-TB among MDR-TB suspects and common mutations in rpoB, katG and inhA genes using GenoType MTBDRplus in the N-E region.

Material & Methods

A total of 553 sputum samples from MDR-TB suspect patients from seven North-Eastern States namely Arunachal Pradesh; 121, Assam; 95, Manipur; 75, Meghalaya; 97, Mizoram 89, Nagaland; 46 and Tripura 30 collected during January 2012 to August 2012 were received in the Microbiology Department of the Institute at New Delhi, India. The samples were collected from the patients in each peripheral designated microscopy centres (DMC) in the above States and transported in cold-chain as per PMDT guidelines to this laboratory. The collection and transport was supervised by the district TB officers (DTOs) in the States. The MDR-suspect patients enrolled under PMDT in these States included TB patients who were either on category I anti-tubercular treatment with fifth month follow up smear positive for acid-fast bacilli (AFB), or category II treatment with four month follow up AFB smear positive or contacts of MDR-TB (Criterion A). The study protocol was approved by institute’s ethical committee.

Sample processing: All specimens were screened for presence of AFB by Ziehl-Neelsen (ZN) staining. The samples were processed by N-acetyl-L-cysteine-Sodium hydroxide (NALC-NaOH) method of digestion and decontamination. All smear positive (> 1+ AFB) sputum samples received within 72 h of sample collection in cold chain were subjected to only LPA after NALC-NaOH processing whereas the remaining samples including smear negative and scanty AFB positive were subjected to culture as per PMDT guidelines.

Culture: For culture, processed samples were inoculated in MGIT 960 tubes. Tubes flashed positive were identified for Mycobacterium tuberculosis by smear microscopy (serpentine cording) and rapid immunochromatographic test (MPT64 TB Ag detection). Cultures positive for M. tuberculosis were subjected to LPA.

Line probe assay: The GenoType MTBDRplus assay (Hain Life Sciences, Nehran, Germany) was carried out as per manufacturer’s instructions. DNA extraction, master-mix preparation, DNA amplification and hybridization were done after thorough cleaning in separate dedicated rooms. For DNA extraction from NaLC-NaOH processed sample and culture, 500 μl and 1 ml aliquots, respectively were taken. Final DNA obtained was subjected to amplification and the rest was stored at - 20°C.

Master-mix was prepared using reagents provided in the kit in 45 μl volume to which 5 μl DNA was added. The PCR products were detected by hybridization using LPA methodology. The result of DNA strips was interpreted with the help of reporting card as resistant or sensitive for RIF and INH. The presence or absence of all wild type and mutant bands was recorded systematically.

The reports were communicated electronically to the TB co-ordinators of the respective States. The LPA on any invalid results were repeated using stored processed sample deposits. In each run, M. tuberculosis H37Rv (ATCC 27294) and sterile molecular grade water were included as positive and negative controls, respectively.

Results

Of the 553 patients, 390 were males and 163 were females with a male:female ratio of 2.4:1. In Tripura, 27 of 30 (90%) of suspects were males. Most patients (424 of 553, 76.7%) belonged to the age group of 15-45 yr which included 224 (40.5%) in the age group 15-29 and 200 (36.2%) in age group 30-45; 113 patients were more than 45 yr whereas 16 were less than 15 years.
The smear microscopy results are given in Table I. The smear positive and negative patients were 372 (67.3%) and 181 (32.7%), respectively. A total of 339 GenoType MTBDRplus were conducted which included 43 culture isolates obtained from smear negatives/scanty positive cultures. The State-wise data of LPA is detailed in Table II. Valid LPA results were obtained for 328 of 339 (97.8%) strains. Of the 328, 181 (55.2%) tests found to be resistant to both RIF and INH, 28 (8.5%) were resistant to RIF only, 29 (8.8%) were resistant to INH only and the remaining 90 (27.4%) were sensitive to both RIF and INH. The total RIF resistant cases diagnosed were 209 (63.7%). The LPA result to these states from cultures and samples could be dispatched in an average of 23.4 days and 5.2 days respectively from reviewing of the sample.

The TAT of DST using LPA was reduced significantly by 93.5 per cent as compared to solid culture DST (9-12 wk).

The mutations responsible for RIF and INH resistance are displayed in Table III. Among 209 RIF resistant isolates, missing WT (wild type) along with known mutations could be detected in 165 (78.9%). Commonest known RIF mutation was in codon S531L (136/209; 65.1%) followed by D516V mutation (15/209; 7.2%), H526Y mutation (12/209; 5.7%) and H526D mutation (4/209; 1.9%). In 44 (21.1%) RIF resistsants, one or more wild type probes were missing with no bands in mutant probes. These 44 included missing WT8 (11; 25 %), missing WT3/ WT4 (9; 20.5%), missing WT2 (7; 15.9%), missing WT7 (5; 10.8%).

### Table I. State-wise distribution of AFB smear positive and negative samples; AFB reporting as per RNTCP guidelines

| States               | Scanty | 1+ | 2+ | 3+ | Total smear positives (%) | Smear negative (%) | Total samples |
|----------------------|--------|----|----|----|---------------------------|-------------------|--------------|
| Arunachal Pradesh    | 10     | 22 | 20 | 15 | 67 (55.4)                 | 54 (44.6)         | 121          |
| Assam                | 9      | 32 | 8  | 23 | 72 (75.8)                 | 23 (24.2)         | 95           |
| Manipur              | 13     | 34 | 4  | 10 | 61 (81.3)                 | 14 (18.7)         | 75           |
| Meghalaya            | 8      | 30 | 12 | 18 | 68 (70.1)                 | 29 (29.9)         | 97           |
| Mizoram              | 7      | 29 | 7  | 14 | 57 (64)                   | 32 (46)           | 89           |
| Nagaland             | 6      | 13 | 4  | 6  | 29 (63)                   | 17 (47)           | 46           |
| Tripura              | 3      | 7  | 4  | 4  | 18 (60)                   | 12 (40)           | 30           |
| North-East States    | 56     | 167| 59 | 90 | 372 (67.3)                | 181 (32.7)        | 553          |

### Table II. Rifampicin and isoniazid susceptibility result using GenoType MTBDRplus

| States             | LPA done | LPA valid | RIF & INH sensitive | Mono-RIF resistance | Mono-INH resistance | MDR | RIF resistant/ LPA done (%) |
|--------------------|----------|-----------|---------------------|---------------------|---------------------|-----|-----------------------------|
| Arunachal Pradesh  | 71       | 69 (97.2)| 12 (16.9)           | 8 (11.3)            | 5 (7.0)             | 44  | 62.0                        | 73.20 |
| Assam              | 52       | 52 (100)| 13 (25.0)           | 4 (7.7)             | 4 (7.7)             | 31  | 59.6                        | 67.20 |
| Manipur            | 50       | 50 (100)| 20 (40.0)           | 3 (6)               | 9 (18.0)            | 18  | 36.0                        | 42    |
| Meghalaya          | 66       | 63 (95.5)| 4 (6.1)             | 5 (7.6)             | 2 (3.0)             | 52  | 78.8                        | 86.40 |
| Mizoram            | 62       | 59 (95.2)| 28 (45.2)           | 6 (9.7)             | 6 (9.7)             | 19  | 30.6                        | 40.30 |
| Nagaland           | 23       | 20 (87.0)| 8 (34.8)            | 0                   | 2 (8.7)             | 10  | 43.5                        | 43.50 |
| Tripura            | 15       | 15 (100)| 5 (33.3)            | 2 (13.3)            | 1 (6.7)             | 7   | 46.7                        | 60.00 |
| North-East States  | 339      | 328 (97.2)| 90 (26.5)          | 28 (8.3)            | 29 (8.8)            | 181 | 53.4                        | 61.70 |

LPA, line probe assay; MDR, resistant to both rifampicin and isoniazid; RIF, rifampicin; INH, isoniazid
Numbers in parentheses denote percentages
### Table III. Pattern of gene mutations detected by GenoType MTBDRplus assay in drug resistant *M. tuberculosis*  

| Gene | Band missing | Gene region | Mutation present | MDR (n=181) | Mono rifampicin resistant (n=28) | Mono isoniazid resistant (n=29) | Total |
|------|--------------|-------------|------------------|-------------|----------------------------------|----------------------------------|-------|
| **rpoB** | WT8 | 530-533 | S531L | 108 | 17 | 125 |
| | WT8 | 530-533 | UK | 9 | 2 | 11 |
| | WT7 | 526-529 | H526Y | 5 | 1 | 6 |
| | WT4 | 516-519 | UK | 0 | 1 | 1 |
| | WT3, WT4 | 513-517, 516-519 | D516V | 11 | 3 | 14 |
| | WT3, WT4 | 513-517, 516-519 | UK | 7 | 2 | 9 |
| | + | S531L* | 8 | 2 | 10 |
| | WT5, WT6 | 518-522,521-525 | UK | 1 | 0 | 1 |
| | WT7 | 526-529 | UK | 5 | 0 | 5 |
| | WT7 | 526-529 | H526D | 2 | 0 | 2 |
| | WT4, WT8 | 516-519, 530-533 | S531L | 1 | 0 | 1 |
| | + | H526Y* | 2 | 0 | 2 |
| | + | H526Y, H526D* | 2 | 0 | 2 |
| | + | H526Y, S531L* | 2 | 0 | 2 |
| | WT1, WT2 | 505-509, 510-513 | UK | 2 | 0 | 2 |
| | WT2 | 510-513 | UK | 7 | 0 | 7 |
| | WT2, WT3, WT4 | 510-513, 513-517, 516-519 | UK | 1 | 0 | 1 |
| | WT2, WT3 | 510-513, 513-517 | UK | 1 | 0 | 1 |
| | WT2, WT7 | 510-513, 526-529 | UK | 1 | 0 | 1 |
| | WT3 | 513-517 | UK | 2 | 0 | 2 |
| | WT3 | 513-517 | D516V | 1 | 0 | 1 |
| | WT3, WT4, WT8 | 513-517, 516-519, 530-533 | UK | 1 | 0 | 1 |
| | WT4, WT5 | 516-519, 518-522 | UK | 1 | 0 | 1 |
| | WT4, WT8 | 516-519, 530-533 | UK | 1 | 0 | 1 |
| **Total** | | | | 181 | 28 | 209 |

| **katG** | WT | 315 | S315T1 | 154 | 22 | 176 |
| | WT | 315 | S315T2 | 0 | 2 | 2 |
| | WT | 315 | UK | 7 | 4 | 11 |
| | + | S315T1* | 3 | 0 | 3 |
| **Total** | | | | 164 | 28 | 192 |

| **inhA** | WT1 | 15 / 16 | C15T | 19 | 1 | 20 |
| | WT1 | 15 / 16 | UK | 2 | 0 | 2 |
| | + | T8C* | 1 | 0 | 1 |
| **Total** | | | | 22 | 1 | 23 |

MDR: resistance to both rifampicin & isoniazid; *Heteroresistant isolates; UK: No known mutations as defined by the kit
11.4%), missing WT1/WT2 (2; 4.5%), missing WT3 (2; 4.5%), missing WT2/WT3 (1; 2.3%), missing WT2/WT3/W4 (1; 2.3%) missing WT2/WT7 (1; 2.3%), missing WT3/WT4/W8 (1; 2.3%), missing WT4 (1; 2.3%), missing WT4/WT5 (1; 2.3%), missing WT4/WT8 (1; 2.3%) and missing WT5/WT6 (1; 2.3%). Mixed pattern to RIF with all wild type probes present along with presence of one or more mutant bands was found in 7.7 per cent (16/209), commonest being S531L (10; 66.7%).

Among 210 INH resistant isolates as detected by MTBDRplus, katG mutations occurred in 192 (91.4%). Mutations in codon S315T1 were detected in 179 (85.2%) of INH resistant or 179 of 192 (93.2%) of katG mutants. Missing wild types with unknown mutant probe among katG were found in 11(5.7%). Mutations in InhA were found in 23 (10.96%) INH resistant isolates, which included 20 (86.95%) C15T and 1 (4.3%) T8C. Both inhA and katG mutations were seen in 5/210 (2.4%) INH resistant isolates. Mixed wild-type and mutant pattern to INH was found in 19 per cent (4/209).

Discussion

Till August 2012, all samples from the region were referred to our institute due to lack of culture and DST or LPA facility. The implementation of TB control in these States is challenging due to poor accessibility, difficult geographical terrains, weak infrastructure and politico-social issues. Males were predominant with 70.5 per cent and majority patients belonged to the young age group of 15-45 yr as is also seen in other studies9. High MDR-TB burden in young adult males have many socio-economic implications. High percentage of MDR-TB burden in young adult males has been reported from France; 62.5 per cent, Uganda; 61.5 per cent and South Africa; 57.6 per cent. In the present study, the test identified RIF resistance by one of four rpoB mutant probes in 78.9 per cent strains, much lesser than in South African study (88.6%)14. Mutation in codon S531L was detected in 65.1 per cent of RIF resistant cases, whereas other Indian studies found it in 59.8 and 84.6 per cent cases15,16.

However, such patients were started on category IV treatment within 1 wk of report communication, thereby curtailing the spread of the diseases.

The GenoType MTBDRplus detects the mutations for RIF and INH, which highly correlate with the sequencing13. In the present study, the test identified RIF resistance by one of four rpoB mutant probes in 78.9 per cent strains, much lesser than in South African study (88.6%)14. Mutation in codon S531L was detected in 65.1 per cent of RIF resistant cases, whereas other Indian studies found it in 59.8 and 84.6 per cent cases15,16. Internationally, S531L mutation has been detected in rates varying from 47 to 70.5 per cent13,14,17,18. In some studies S531L mutation occurred more frequently in MDR isolates in comparison to RIF mono-resistant14,17. However, we did not find any such difference. The missing wild type probe without any mutant bands was found in 21.1 per cent of RIF resistant cases as also reported in other studies, New Delhi; 11.1 per cent, Vietnam; 33.3 per cent, France; 29 per cent, Uganda; 42.1 per cent14,18,3,17. In GenoType MTBDRplus the entire wild type region is covered by wild type probes whereas only common mutant probes are covered. As per the kit insert provided, absence of any wild type band along with presence or absence of mutant band, accounts for resistance. Such isolates with uncommon mutations could have been identified by sequencing. One limitation of the study was the inability to perform sequencing due to lack of such set up in this facility.

katG mutations account for commonest mechanism of resistance for INH. Specific mutation in codon S315T1 of katG was found in 86.2 per cent of INH resistant isolates, similar to other studies17,19,21. Variations have been reported from France; 62.5 per cent, Uganda; 61.5 per cent and South Africa; 37.6%17,14. High prevalence of katG mutations accounts for more INH resistance in high burden countries and high-level INH resistance19.

Mutation in inhA gene accounts for 15-20 per cent of all INH resistant cases and low-level resistance. It was found to be 11.0 per cent, similar to studies elsewhere5.4 to 21.1 per cent3,17,18,20. However, Barnard et al14 reported high prevalence of inhA mutations (41.7%). They also found significant difference in prevalence of mutations in MDR strains as compared to mono-INH resistant14. We did not find any such difference.

Heteroresistance defined as presence of all wild type probes along with presence of one or more mutant bands was found in 7.7 per cent of RIF resistant and
in 1.9 per cent of INH resistant isolates unlike a study from Mumbai\(^21\). A disadvantage of any genotypic test is a possibility of silent mutation, \(\text{i.e.}\) mutations which do not lead to change in amino acid hence not leading to phenotypic drug resistance. Resistance originating from mutation of other genes as well as other resistant mechanisms will not be detected by this test. Such mutations could be detected by sequencing.

In conclusion, our study shows the resistance to RIF and INH among \(M.\) \textit{tuberculosis} isolates obtained from MDR-TB suspects of North-Eastern region of India using the GenoType MTBDR\textit{plus} assay. In addition, information on the common mutations in the \(rpoB,\) \(katG\) and \(inhA\) regions associated with resistance in also provided.

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