Association of gyrA and rrs gene mutations detected by MTBDRsl V1 on Mycobacterium tuberculosis strains of diverse genetic background from India

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There is limited data on the use of Genotype MTBDRsl Version 1 (MTBDRsl_V1) as an initial rapid screening test to rule out XDR-TB and most importantly its performance in various genotypes of Mycobacterium tuberculosis is scarcely studied. A total of 359 MDR-TB isolates were tested for gene mutations representing second line drug resistance, using the MTBDRsl_V.1 and the results were compared with phenotypic method (Bactec MGIT-960 system) for second-line drug (SLD) susceptibility testing. Genetic lineages of all these isolates were also determined using spoligotyping and SITVIT2 WEB database. The MTBDRslV1 detected mutations in the gyrA, rrs, and emb genes in 108 (30%), 2 (0.5%) and 129 (35.9%) isolates, respectively. Remaining 120 (33.4%) had no second line drug (SLD) resistance. In 17 (4.7%) isolates mutations were detected in both gyrA and rrs genes. Its concordance with MGIT-960 culture drug susceptibility testing (DST) was 97% and 94.1%, 93.5%, 60.5% and 50% for the detection of XDR-TB, pre-XDR, Ethambutol, and Aminoglycosides/Cyclopeptides resistance. The Beijing lineage was predominant (46%) between both the pre-XDR/XDR-TB isolates. We conclude that MTBDRsl is useful for rapid detection of SLD resistance. Also in pre-XDR and XDR-TB isolates the frequency of relevant genetic mutations was significantly higher in the Beijing strains.

The recent estimates of World Health Organization (WHO) show that more than 10.4 million people had incident tuberculosis (TB) and 1.7 million died of this disease in 2016 alone. The 2016 data also showed that India is the most vulnerable country to TB with 2.8 million new cases (26.9% of global cases) in the year 2016. The scenario was not much different in 2017. Approximately 600,000 Rifampicin resistant (RIFr3) cases were reported, of whom 490,000 had multi-drug resistant TB (MDR-TB). The drug resistance TB has become a major challenge to the success of TB control programmes around the globe7. Not only diagnosis, even the treatment of MDR-TB is difficult, requiring much longer duration of treatment with very costly and comparatively more toxic second-line drugs (SLDs) such as fluoroquinolones (FQ) and aminoglycosides/cyclic peptides (AG/CP)5. Moreover, emergence of resistance even to the SLDs is being reported from all parts of the world. This form of TB is known as extensively drug-resistant TB (XDR-TB), which is a more devastating condition with very poor treatment success7. In 2016, an estimated 6.2% patients with MDR-TB were found to have XDR-TB, India reported first case of XDR-TB in 2007 and by 2015 more than 117 countries were already struggling with this condition5. Therefore, it is essential that all suspected cases of MDR-TB must be investigated for susceptibility to second drugs also, in a timely manner to control the spread of XDR-TB. This has become essential in order to optimize the treatment regimen at the earliest. However, the conventional methods of drug susceptibility testing (DST) for SLDs are more complex due to non-standardized methods and protocols, leading to poor reproducibility and reliability9. Also, the conventional culture-based methods are labour intensive and require longer turn around time (TAT) with undesirable treatment outcome and wider window to disease transmission7,8.
In 2013, a Line probe assay (LPA), also known as Genotype MTBDRsl (MTBDRsl) was developed by Hain’s Life Science GmbH, Germany and was approved by the WHO also for clinical use. This molecular test rapidly detects genotypic resistance to FQ, AG/CP and Ethambutol (EMB) within 48–72 h and makes it possible to diagnose pre-XDR-TB and XDR-TB at the earliest[10]. Therefore, the use of MTBDRsl has been recommended as a rapid and initial diagnostic test to rule out FQ and AG/CP resistance in all MDR-TB patients in order to initiate effective treatment at the earliest[11]. Recently, WHO recommended the use of MTBDRsl test in place of conventional phenotypic culture-based DST methods especially in high TB burden countries[12]. India, a high TB burden country is keen to implement these guidelines but usefulness of MTBDRsl on MDR TB isolates has not yet been evaluated from India.

The genotypic characterization of MTB isolates becomes essential to understand the clonal expansion of the lineages, their transmission dynamics and association with drug resistance[13]. There are several studies which have shown association of different lineages of MTB with variable pathogenicity and vulnerability to drug resistance[14,15]. For genotyping of the MTB, spoligotyping is a widely used technique which detects the presence or absence of 43 spacer sequences in clustered regularly interspersed short palindromic repeat (CRISPR) region of MTB[16]. Though several studies are published on association of various lineages of MTB with first-line drug resistance, there is not enough literature on the association of MTB lineages with resistance to second-line drugs[14].

Thus, the main aim of the present study was to evaluate the usefulness of MTBDRsl assay in comparison with phenotypic line drug resistance testing using the Bactec MGIT-960 system in a programmatic mode. We also aimed to find if there was any association between the second line drug resistance pattern and genetic lineages of MTB isolates.

Table 1. Showing association of lineages with place of origin, smear positivity, site of isolation and drug resistance. PTB- Pulmonary Tuberculosis. EPTB- Extra-pulmonary Tuberculosis.

| Main Residence of patient | Smear microscopy | Site of sample | Drug resistance Pattern | Genotypes |
|---------------------------|-----------------|----------------|-------------------------|-----------|
|                           | Smear +ve        | Smear -ve       | Pre-XDR                  | XDR       |
| Assam (44)                | 44              | 0              | 20                      | 21        |
| Bihar (35)                | 12              | 23             | 15                       | 20        |
| Delhi (132)              | 61              | 71             | 74                       | 58        |
| Haryana (5)              | 1               | 4              | 2                        | 3         |
| J&K (1)                  | 0               | 1              | 0                        | 1         |
| Meghalaya (20)           | 20              | 0              | 20                       | 0         |
| Mizoram (2)              | 0               | 2              | 2                        | 2         |
| Madhya Pradesh (1)       | 0               | 1              | 0                        | 1         |
| Punjab (70)              | 70              | 0              | 70                       | 42        |
| Rajasthan (1)            | 0               | 1              | 0                        | 1         |
| Sikkim (10)              | 8               | 2              | 8                        | 2         |
| Tripura (13)             | 13              | 0              | 13                       | 0         |
| Uttar Pradesh (24)       | 11              | 13             | 11                       | 13        |
| Uttarakhand (1)          | 0               | 1              | 0                        | 1         |
| Total (359)              | 240             | 119            | 259                      | 100       |

In-vitro subculture of the All India Institute of Medical Sciences, New Delhi (reference number IESC/T-39/04.01.2013). All in-vitro methods were performed in accordance with the standard guidelines and following the manufacturer's instructions. The mycobacterial culture and DST were performed using the MGIT-960 system and identification of MTB isolates was made by in-house multiplex polymerase chain reaction (PCR)[19].

Demographic details and characteristics of MDR-TB patients. Out of the 359 patients from whom these isolations were made, 210 (58.5%) were males and 149 (41.5%) females with mean age of 31.4 ± 14.3 years and 27.1 ± 15.8 years, respectively. Majority of cases were adults [331 (92.2%)] and only a few [28(7.8%)] were from paediatric age group. Majority of isolates [259 (72.1%)] were from pulmonary samples and 100 (27.8%) isolates were from extra-pulmonary samples (Supplementary Fig. 1). A total of 240 (66.8%) isolates were from smear positive while 119 (33.2%) isolates were from smear negative samples (Table 1).

Subculture on Lowenstein Jensen (L-J) medium for pure growth. From each Bactec MGIT-960 tube, 200 μL of culture suspension was sub-cultured on L-J medium slants and incubated at 37°C to obtain pure...
growth (isolated colonies) of MTB. After 21–28 days of incubation, a single colony with the help of sterile inoculating loop was picked from L-J medium and inoculated in the MGIT (Mycobacteria growth indicator tube). The tube was incubated in the Bactec MGIT-960 system until flagged positive and this growth was used for second-line MGIT DST and DNA extraction for spoligotyping and MTBDR<sup>sl</sup>.

Preparation of drug stock and working solutions. All SLDs were purchased from Sigma-Aldrich Corporation (St. Louis MO, USA) in the form of powder. The stock solutions of amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) were prepared in sterile deionized water while ofloxacin (OFX) solution was prepared in 0.1N-NaOH. Stock solutions were sterilized through 0.22-μM-pore-size Milex-GS filter units (Millipore Bedford MA, USA) and the aliquots stored at −80 °C for further use.

Second Line Drug susceptibility (DST) testing using Bactec MGIT-960 system. Aliquots of OFX, AMK, KAN and CAP stock solutions were diluted to critical concentrations as recommended to perform the second line DST by Bactec MGIT-960 system<sup>7</sup>. DST was performed on Day 1 and Day 2 by single dilution [0.5 mL of 1:100 dilution inoculum for growth control (GC) and 0.5 mL of inoculums directly in four respective drug containing tubes] while from the growth of Day 3 to Day 5 by double dilution [inoculated 0.5 mL of 1:4 dilution inoculums directly into four drug containing tubes] and in GC tubes using the 1:100 dilution of the inocula from the day MGIT flashed positive. GC tubes and four drug panel tubes were set in the antimicrobial susceptibility testing (AST) carrier rack and loaded in the Bactec MGIT-960 system and continuously monitored by BD Epi-center<sup>21</sup>. As AST carrier rack for SLDs, the panel is not available commercially for the MGIT-960 system, it was registered as one of the SIRE (Streptomycin, Isoniazid, Rifampicin, Ethambutol) panel in order to get a printable report and drug susceptibility testing results<sup>22</sup>.

Genotype MTBDR<sup>sl</sup>V1. The MTBDR<sup>sl</sup> test is based on the DNA strip technology having three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. All steps were performed as per manufacturer’s instructions<sup>10,23</sup>. The DNA obtained from the standard MTB-<i>H37Rv</i> strain (as positive control) and one negative control was also tested in each batch in order to check the cross-contamination during hybridization assay and other quality parameters. The test was considered as valid; only when the hybridization bands were obtained on MTB complex control (TUB), conjugate controls (CC) and the amplification controls (AC) along with the targeted gene loci controls. For convenience MTBDR<sup>sl</sup>V1 will be referred as MTBDR<sup>sl</sup> only hereafter.

Genotyping of MDR-TB isolates by spoligotyping. DNA extraction for spoligotyping. DNA extraction from MTB cultures grown on L-J medium was performed using chloroform iso-amyl alcohol (CI) method as mentioned previously<sup>24</sup>.

Spoligotyping. Spoligotyping was performed using the commercially available kit (Ocimum Biosolutions, Hyderabad, India) by amplification, hybridization and finally detection of hybridizing DNA<sup>25</sup>. In brief, the PCR amplified products were hybridized on a membrane and images were detected with electro-chemi-luminescence (ECL) detection kit (GE Healthcare, Life Sciences, UK) on X-ray films (Kodak, Rochester, NY)<sup>14</sup>. The MTB

Figure 1. The figure shows minimum spanning tree of MDR-TB (but sensitive to second-line drug) isolates characterized by spoligotyping. Each circle represents a genotype. The distance between circles represents how closely related are different genotypes to each other.
and M. bovis-BCG strains were included as a quality control in every batch of test. The hybridization patterns obtained in the binary format were transformed to an octal code for assessment with the spoligotype patterns using SITVIT2 database, which is an updated version of SITVIT_WEB database. A shared type was defined as a spoligotype pattern common to at least two isolates, and clades were assigned according to the signatures as published earlier.

Statistical analysis. Results of MTBDR<sub>sl</sub> were analysed and compared with second-line Bactec MGIT-960 DST, which was considered as the gold standard. Data was statistically analysed to calculate the agreement between MTBDR<sub>sl</sub> and SL-MGIT DST using OpenEpi 3.01. Moreover, Fisher's exact test was performed using STATA 11.1 software to observe significance of the association amongst the second line drug resistant mutation patterns among the different genotypes.

Results

Second-line DST by Bactec MGIT-960 system. Using the phenotypic MGIT-960 system, of the 359 MDR-TB isolates subjected to second-line DST, 231 (64.4%) were found to be susceptible to all second line anti-TB drugs, 127 had resistance to FQ. Of the 127 isolates, 110 (30.6%) were mono-resistant to FQ (pre-XDR)
and 10 (2.7%) isolates were resistant to all OFX-KAN-AMK-CAP. 6 (1.7%) were resistant to OFX-KAN-AMK and 1 (0.3%) isolate was resistant to OFX-KAN only. Thus, phenotypically these 17 (4.7%) isolates were labelled as XDR-TB isolates. Only one (0.3%) isolate was resistant to AG/CP (Table 2).

EMB resistance. All 359 isolates previously tested for SIRE DST, 143 (39.8%) isolates were detected as resistant and 216 (60.2%) isolates were detected sensitive to EMB (Supplementary Table 1).

Genotype MTBDRslV1. Out of 359 MDR-TB isolates, in 232 (64.6%) isolates no mutation was detected in gyrA and rrs genes, which means that these were sensitive to FQ and AG/CP drugs. However, 125 (34.8%) isolates showed mutations in gyrA region, of which 108 showed FQ mono-resistant (pre-XDR) but 17 also showed additional mutations in rrs genes (XDR-TB) (Table 2). Among the single codon mutations observed in gyrA region, the most prevalent mutation was ΔWT3-D94G (51; 40.8%) followed by ΔWT3-A90V (31; 24.8%). However, among the double codon mutations, the most prevalent mutation was A90V-D94G (7; 5.6%) followed by ΔWT3-D94G-D94H (2; 1.6%). Only one strain harboured triple codon mutation ΔWT3-D94N/Y-D94H-D94G in gyrA region (Table 3).

Of the 19 (15.2%) isolates that showed resistance mutation pattern in the rrs region, 17 (89.4%) were XDR-TB and 2 (10.6%) were AG/CP mono-resistant. In these isolates, most prevalent mutation was ΔWT1-A1401G (11; 57.9%) and A1401G (31.6%). The detailed mutational patterns in gyrA and rrs genes are shown in Table 3.

Detection of EMB resistance by Genotype MTBDRslV1 in emb gene. Overall MTBDRsl detected 129 (35.9%) isolates having resistance to EMB. Of these 58 (44.6%) isolates were mono-resistant to FQ, 1 (0.7%) isolate was mono-resistant to AG/CP only and 9 (6.9%) isolates were XDR-TB isolates. The most prevalent mutations in the emb gene were ΔWT1-Mut1b in 95 (73.6%) isolates, ΔWT1-Mut1a in 20 (15.5%), ΔWT in 10 (7.7%), and ΔWT1-Mut1a-Mut1b in 4 (3.1%) isolates.

Comparison of second line DST using MGIT-960 versus Genotype MTBDRslV1. The MTBDRsl showed 97% concordance with second-line MGIT-960 DST for detecting the sensitive isolates. However, mutations were detected in 101 (93.5%) isolates that were FQ resistant by MGIT-960 system. MTBDRsl also efficiently detected 17 (4.7%) isolates as XDR-TB giving a concordance rate of 94.1% with MGIT-960 system (Table 2). Of the 143 (39.8%) EMB resistant isolates identified by SIRE MGIT-960 DST, 86 (60.1%) were detected resistant and 57 (39.9%) as sensitive by the MTBDRsl. Thus, the overall concordance of MTBDRsl with MGIT-960 system was only 50% for detection of EMB resistance.

Genotyping Results. The spoligotyping results showed that 142 (39.5%) isolates belonged to CAS lineage, 119 (33.2%) Beijing, 26 (7.2%) T, 13 (3.6%) EAI, 12 (3.3%) Haarlem, 8 (2.2%) X, 7 (1.9%) Manu and 2 (0.5%) isolates each of Ural and S lineages. Twenty-eight (7.7%) isolates showed Unique (U) patterns. Out of 359 MDR-TB isolates, 322 (89.7%) were grouped in 55 STs (Shared international types). Nine (2.5%) strains were identified as “orphans”. The remaining 28 (7.7%) isolates could not be categorized in any STs (Table 4). Among the 55 STs...
| SIT | SIT DESCRIPTION | OCTAL CODE | LINEAGE | RESISTANT TYPE  |
|-----|----------------|------------|---------|-----------------|
|     |                |            |         | MDR (N = 231)   | Pre-XDR (N = 111) | XDR (N = 17) | Total (%) |
| 1   |                | 000000000003771 | BEIJING | 57 (24.7)       | 46 (41.4)         | 8 (47.1)    | 111 (30.9) |
| 11  |                | 47777777413071 | EAI3_IND | 4 (1.7)         | 1 (0.9)           | 0           | 5 (1.4)    |
| 25  |                | 703777740003171 | CASI DELHI | 11 (4.7)   | 1 (0.9)           | 0           | 12 (3.3)   |
| 26  |                | 703777740003771 | CASI DELHI | 65 (28.1)  | 18 (16.2)         | 1 (5.8)     | 84 (23.4)  |
| 27  |                | 703777747770371 | URAL     | 0             | 1 (0.9)           | 0           | 1 (0.3)    |
| 34  |                | 77637777760771 | S         | 2 (0.9)        | 0                 | 0           | 2 (0.6)    |
| 37  |                | 77773777760771 | T3        | 1 (0.4)        | 0                 | 0           | 1 (0.3)    |
| 48  |                | 77777777413731 | EAI2_SOM  | 0             | 2 (1.8)           | 0           | 2 (0.6)    |
| 52  |                | 7777777760731 | T2        | 1 (0.4)        | 0                 | 0           | 1 (0.3)    |
| 53  |                | 7777777760771 | T1        | 9 (3.9)        | 0                 | 0           | 9 (2.5)    |
| 54  |                | 7777777763771 | MANU2     | 2 (0.9)        | 2 (1.8)           | 0           | 4 (1.1)    |
| 67  |                | 77777703720771 | H3        | 4 (1.7)        | 1 (0.9)           | 0           | 5 (1.4)    |
| 92  |                | 70007677760771 | X3        | 1 (0.4)        | 0                 | 0           | 1 (0.3)    |
| 100 |                | 7777777773771 | MANU1     | 1 (0.4)        | 0                 | 0           | 1 (0.3)    |
| 119 |                | 77776777760771 | X1        | 0             | 4 (3.6)           | 1 (5.8)     | 5 (1.4)    |

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| SIT | SIT DESCRIPTION | OCTAL CODE | LINEAGE | RESISTANT TYPE | MDR (N = 231) | Pre-XDR (N = 111) | XDR (N = 17) | Total (%) |
|-----|----------------|------------|---------|----------------|--------------|-------------------|-------------|-----------|
| 125 | **                | 000000007760731 | T2 | 0 | 1 (0.9) | 0 | 1 (0.3) |
| 127 |                  | 57777777420771 | H4 | 2 (0.9) | 0 | 0 | 2 (0.6) |
| 137 |                  | 77777677760601 | X2 | 0 | 1 (0.9) | 0 | 1 (0.3) |
| 190 | **                | 00000000003731 | BEIJING | 0 | 2 (1.8) | 0 | 2 (0.6) |
| 236 |                  | 77777777413771 | EAI5 | 0 | 0 | 1 (5.8) | 1 (0.3) |
| 243 |                  | 77777777760600 | T1 | 2 (0.9) | 2 (1.8) | 0 | 4 (1.1) |
| 250 |                  | 00000000000371 | BEIJING | 2 (0.9) | 0 | 0 | 2 (0.6) |
| 283 |                  | 777777770420771 | H1 | 3 (1.3) | 1 (0.9) | 0 | 4 (1.1) |
| 288 |                  | 700377774003771 | CAS2 | 1 (0.4) | 1 (0.9) | 0 | 2 (0.6) |
| 289 |                  | 703777774003571 | CASI DELHI | 2 (0.9) | 0 | 0 | 2 (0.6) |
| 344 |                  | 70807777760771 | T1 | 1 (0.4) | 0 | 0 | 1 (0.3) |
| 357 |                  | 7037777740000771 | CAS | 3 (1.5) | 0 | 0 | 3 (0.8) |
| 358 | **                | 71777777760771 | T1 | 0 | 1 (0.9) | 0 | 1 (0.3) |
| 428 |                  | 703777740003371 | CASI DELHI | 2 (0.9) | 0 | 0 | 2 (0.6) |
| 429 |                  | 703777740003731 | CASI DELHI | 0 | 1 (0.9) | 1 (5.8) | 2 (0.6) |

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| SIT | SPOLIGOTYPE DESCRIPTION | OCTAL CODE | LINEAGE | RESISTANT TYPE | MDR (N = 231) | Pre-XDR (N = 111) | XDR (N = 17) | Total (%) |
|-----|--------------------------|------------|---------|----------------|---------------|-------------------|--------------|-----------|
| 462 | T1                       | 7777777560771 | Ural    | 1 (0.4)        | 0             | 0                 | 1 (0.3)      | 1 (0.3)   |
| 464** | URAL                    | 7777777747771 | EU-276  | 0              | 0             | 1 (5.8)           | 1 (0.3)      | 1 (0.3)   |
| 486 | CAS                      | 70377774000371 | CAS     | 2 (0.9)        | 1 (0.9)      | 0                 | 3 (0.8)      | 3 (0.8)   |
| 591 | EA6_BGD1                 | 7777775741371 | CAS1    | 2 (0.9)        | 0            | 0                 | 2 (0.6)      | 2 (0.6)   |
| 621 | BEIJING                  | 00000000002771 | BEIJING | 0              | 0            | 1 (5.8)           | 1 (0.3)      | 1 (0.3)   |
| 754 | CASI DELHI               | 50377774000371 | CASI    | 0              | 2 (1.8)      | 0                 | 2 (0.6)      | 2 (0.6)   |
| 794 | CASI DELHI               | 70375774000371 | CASI_Delhi | 0      | 2 (1.8)      | 0                 | 4 (1.1)      | 4 (1.1)   |
| 798** | T1                      | 43777777760771 | T1      | 0              | 1 (0.9)      | 0                 | 1 (0.3)      | 1 (0.3)   |
| 1091 | CASI DELHI               | 703777740002771 | CASI_Delhi | 1 (0.4)    | 1 (0.9)      | 0                 | 2 (0.6)      | 2 (0.6)   |
| 1092 | CASI DELHI               | 702777740003771 | CASI_Delhi | 1 (0.4)    | 0           | 0                 | 1 (0.3)      | 1 (0.3)   |
| 1120 | CAS                      | 703760000000331 | CAS     | 2 (0.9)        | 0            | 1 (5.8)           | 3 (0.8)      | 3 (0.8)   |
| 1166** | T1                      | 77737777760771 | T1      | 0              | 1 (0.9)      | 0                 | 1 (0.3)      | 1 (0.3)   |
| 1168' | BEIJING                 | 00000000003631 | BEIJING | 3 (1.3)        | 0            | 0                 | 3 (0.8)      | 3 (0.8)   |
| 1327 | CASI DELHI               | 703637740003771 | CASI    | 1 (0.4)        | 2 (1.8)      | 0                 | 3 (0.8)      | 3 (0.8)   |
| 1343 | CASI DELHI               | 703737740003771 | CASI_Delhi | 2 (0.9)    | 0           | 0                 | 2 (0.6)      | 2 (0.6)   |

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| SIT | SPOLIGOTYPE DESCRIPTION | OCTAL CODE | LINEAGE | RESISTANT TYPE | MDR (N = 231) | Pre-XDR (N = 111) | XDR (N = 17) | Total (%) |
|-----|--------------------------|------------|---------|----------------|----------------|-------------------|--------------|-----------|
| 1394 |                          | 77776777760671 | X1      |                | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| 1401 |                          | 70377774003671 | CASI DELHI |            | 3 (1.3)         | 0                 | 0            | 3 (0.8)   |
| 1628 |                          | 077737777413771 | EA5    |                | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| 1789 |                          | 70377740000171 | CAS     |                | 2 (0.9)         | 0                 | 0            | 2 (0.6)   |
| 1877 |                          | 73737777760771 | T1      |                | 0               | 1 (0.9)           | 1 (1.8)      | 2 (0.6)   |
| 1942 |                          | 70337740003771 | CASI DELHI |            | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| 1970 |                          | 77776757413771 | EA5_BGD |                | 2 (0.9)         | 0                 | 0            | 2 (0.6)   |
| 2147 |                          | 70377740003011 | CASI DELHI |            | 0               | 2 (1.8)           | 0            | 2 (0.6)   |
| 2364 |                          | 70360174003771 | CASI DELHI |            | 3 (1.3)         | 0                 | 0            | 3 (0.8)   |
| 2419 |                          | 703000000000371 | CAS     |                | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| ORPHAN 1 |                        | 700000000000771 | CAS |                | 2 (0.9)         | 0                 | 0            | 2 (0.6)   |
| ORPHAN 2 |                        | 70377740003761 | CASI DELHI |            | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| ORPHAN 3 |                        | 70377747760371 | T1      |                | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| ORPHAN 4 |                        | 4777777420771 | H4      |                | 1 (0.4)         | 0                 | 0            | 1 (0.3)   |
| ORPHAN 5 |                        | 77377777763771 | MANU    |                | 0               | 2 (1.8)           | 0            | 2 (0.6)   |

Continued
| SIT    | SPOLIGOTYPE DESCRIPTION | OCTAL CODE   | LINEAGE | RESISTANT TYPE | MDR (N = 231) | Pre-XDR (N = 111) | XDR (N = 17) | Total (%) |
|--------|-------------------------|--------------|---------|----------------|---------------|-------------------|--------------|-----------|
| ORPHAN 6’ |                         | 377777777760601 | T1      | 2 (0.9)        | 0             | 0                 | 2 (0.6)     |           |
| UNIQUE  |                         | 701777740037771 | UNIQUE   | 2 (0.9)        | 0             | 0                 | 2 (0.6)     |           |
| UNIQUE  |                         | 600000377740271 | UNIQUE   | 0              | 1 (0.9)       | 1 (1.8)          | 2 (0.6)     |           |
| UNIQUE  |                         | 760777777760771 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 777767074137771 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 703317400037771 | UNIQUE   | 2 (0.9)        | 1 (0.9)       | 0                 | 3 (0.8)     |           |
| UNIQUE  |                         | 477737777413071 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 777737777620800 | UNIQUE   | 1 (0.4)        | 1 (0.9)       | 0                 | 2 (0.6)     |           |
| UNIQUE  |                         | 777772635607771 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 700377700001751 | UNIQUE   | 2 (0.9)        | 0             | 0                 | 2 (0.6)     |           |
| UNIQUE  |                         | 703731740003777 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 77777601413771 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 003677740000771 | UNIQUE   | 1 (0.4)        | 0             | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 77777704020731 | UNIQUE   | 0              | 1 (0.9)       | 0                 | 1 (0.3)     |           |
| UNIQUE  |                         | 703000000000771 | UNIQUE   | 0              | 1 (0.9)       | 0                 | 1 (0.3)     |           |
(share types), the two most common were ST1 (Beijing) [111 (30.9%)] and ST26 (CAS1_DELHI) [84 (23.4%)] followed by ST25 (CAS1_DELHI, 3.3%) and ST53 (T1, 2.5%). The remaining SITs represented less than 2.5% isolates. Out of 9 orphans, 2 (0.5%) each belonged to CAS, T1, and Manu lineage and 1 (0.3%) each isolate belonged to CAS1_DELHI, T1, and H4 families (Table 4).

**Frequency of various Genotypes in second line drug sensitive isolates.** Out of the 231 (64.3%) isolates that were found sensitive to second line drugs, 108 (46.7%) belong to the CAS lineage, 62 (26.8%) to Beijing and 18 (7.7%) to the T lineage. Seventeen (7.3%) isolates showed Unique SIT patterns, 10 (4.3%) belonged to Haarlem, 9 (3.8%) EAI, 3 (1.3%) Manu and 2 (0.8%) each belong to X and S lineages. While analysing the clustering of these strains, 207 (89.6%) strains could be grouped under 39 STs, while 7 (3.4%) were identified as orphans and the rest 19 (8.2%) had unique patterns (Tables 4 and 5). Among the 39 SITs the commonest were SIT26 (CAS1_DELHI), SIT1 (Beijing) and SIT25 (CAS1_DELHI) with 65 (28.1%), 57 (24.7%) and 11 (4.7%) strains each group. Other genotypes representing fewer genotypes are given in the Table (4).

**Frequency of various Genotypes in pre-XDR-MTB isolates.** Out of 111 (30.9%) pre-XDR TB isolates detected by MGIT-960 DST system, the most frequent genotypes belonged to Beijing (48, 43.2%) genotype, CAS (31, 27.9%) and Unique (10, 9.1%). Other genotypes were less commonly seen and are given in Table (4). Among the 26 SITs identified, like second line drug susceptible isolates, in pre-XDR isolates also, the SIT1 (Beijing) [46; 41.4%] and SIT26 (CAS1_DELHI) [18; 16.2%] were the two most common types followed by Haarlem, 3 (2.7%) and 2 (1.8%) respectively. But the predominance of Beijing genotype was unequivocal (26.8% v 43.2%). Of the two Orphans identified, both belonged to the CAS1_DELHI genotype. The remaining SITs represented less than 3.6% isolate (Supplementary Fig. 2).

**Frequency of various Genotypes of XDR-MTB isolates.** Out of 17 (4.7%) XDR-TB isolates that were detected by the SLD MGIT-960 DST, 9 (52.9%) isolates represented as Beijing lineage, 3 (17.6%) as CAS and 1 (5.8%) each belong to EAI, X, T, Ural, and Unique patterns. Therefore, there was a clear pattern of Beijing genotype become more and more and common in isolates developing drug resistance. A total of 9 SITs were identified among 16 (94.1%) strains and one (5.8%) strain had unique spoligotype pattern. But the predominance of Beijing genotype was unequivocal (26.8% v 43.2%). Of the two Orphans identified, both belonged to the CAS1_DELHI genotype. The remaining SITs represented less than 3.6% isolate (Supplementary Fig. 2).

**Cluster analysis.** The cluster analysis revealed that our isolates belonged to overall 47 clusters with the size of 2–65 isolates in each cluster. The highest clustering (31 clusters) was found among the MDR-TB isolates but sensitive to second-line drugs. Amongst the pre-XDR-TB isolates highest number of clusters were observed in the CAS lineage having 5 clusters (2–18 isolates in each cluster) followed by Beijing with 2 clusters of 2–46 isolates. However, in the XDR-TB isolates only one cluster was found with 8 isolates all belonging to the Beijing lineage. Isolates showing unique patterns were also found in a single cluster with two in the pre-XDR-TB isolates. Interestingly, most of the X, T, orphan and unique strains were un-clustered (Table 5).

Minimum Spanning Tree (MST) analysis done by using the MIRU-VNTRplus software, revealed various SITs amongst different regions showing predominant SITs and evolutionary relationship of the lineages and their SITs. MST connects each genotype based on the degree of changes required to go from one allele to another.
The length of the branches denotes the distance between any two patterns whereas the intricacy of the lines indicates the number of spacers between the two patterns. The thicker lines represent 1 change while thinner ones indicate 2 or 3. The size of the circle is comparative to the total number of MTB isolates in this study. The colour of the circles represents the phylogenetic lineage to which the specific pattern belongs. Beijing patterns are circled in red and yellow indicates CAS strains. EAI strains are in dark green colour while EAI strains are in dark green colour. The Clustering and MST of MDR-TB isolates (but susceptible to second line drugs) is shown in Fig. (1). The clustering details for pre-XDR strains are given in Fig. (2). Out of 111 isolates, a total of 88 (79.3%) were grouped into 13 clusters, whereas 23 (20.7%) were non-clustered isolates of which 8 (34.8%) were unique non-clustered isolates. In case of XDR-TB strains these details are shown in Fig. (3). Out of 17 isolates, 8 (47.1%) isolates could be grouped only in 1 cluster, whereas 9(52.9%) were non-clustered isolates of which 1 (11.1%) was unique non-clustered isolate. Evolution of new share types among MDR-TB isolates. In our study 18 new SITs, were found in MDR-TB isolates, which have not been previously reported so far from India in the SITVIT2 database. Among the second line drug susceptible isolates, SIT 250 (Beijing, n = 1); SIT 462 (T1, n = 1); SIT 1628 (EAI5, n = 1); SIT 1168 (Beijing, n = 3); SIT 1394 (X1, n = 1); SIT 1401 (CAS1_DELHI, n = 3); SIT 2419 (CAS, n = 1); Orphan1 (CAS, n = 2); Orphan2 (CAS1_DELHI, n = 1); Orphan3 (T1, n = 1); Orphan 6 (T1, n = 2) were found to be evolved. New SITs, which were common in both MDR, and pre-XDR-TB isolates, included SIT 67 (H3, n = 5), SIT 243 (T1, n = 1) and SIT 794 (CAS, n = 4), respectively. Amongst the pre-XDR TB isolates, SIT 27 (Ural, n = 1); SIT 125 (T2, n = 1); SIT 190 (Beijing, n = 2); SIT 358 (T1, n = 1); SIT 798 (T1, n = 1); SIT 2147 (CAS, n = 3).

| LINEAGE | MDR | Pre-XDR | XDR | TOTAL |
|---------|-----|---------|-----|-------|
| BEIJING |     |         |     |       |
| Clustered | 3(2–57) | 2(2–46) | 1(10) | 6(2–57) |
| Un-clustered | 0 | 0 | 1 | 1 |
| CAS |     |         |     |       |
| Clustered | 11(2–65) | 5(2–18) | 0 | 16(2–65) |
| Un-clustered | 7 | 5 | 3 | 15 |
| EAI |     |         |     |       |
| Clustered | 3(2–4) | 1(2) | 0 | 4(2–4) |
| Un-clustered | 1 | 1 | 1 | 3 |
| MANU |     |         |     |       |
| Clustered | 1(2) | 1(2) | 0 | 2(2) |
| Un-clustered | 1 | 0 | 0 | 1 |
| H |     |         |     |       |
| Clustered | 3(3–4) | 1(3) | 0 | 4(3–4) |
| Un-clustered | 1 | 1 | 0 | 2 |
| X |     |         |     |       |
| Clustered | 0 | 0 | 0 | 0 |
| Un-clustered | 2 | 1 | 1 | 4 |
| T |     |         |     |       |
| Clustered | 2(2–9) | 1(2) | 1(1) | 4(2–9) |
| Un-clustered | 4 | 5 | 0 | 9 |
| S |     |         |     |       |
| Clustered | 1(2) | 0 | 0 | 1(2) |
| Un-clustered | 0 | 0 | 0 | 0 |
| URAL |     |         |     |       |
| Clustered | 0 | 0 | 0 | 0 |
| Un-clustered | 0 | 1 | 1 | 2 |
| ORPHAN |     |         |     |       |
| Clustered | 2(2) | 1(2) | 0 | 3(2) |
| Un-clustered | 3 | 0 | 0 | 3 |
| UNIQUE |     |         |     |       |
| Clustered | 3(2) | 1(2) | 0 | 6(2) |
| Un-clustered | 7 | 6 | 1 | 14 |
| Total |     |         |     |       |
| Clustered | 31(2–65) | 14(2–46) | 2(9) | 47(2–65) |
| Un-clustered | 26 | 20 | 8 | 54 |

Table 5. Distribution and clustering pattern of MDR-TB isolates.
n = 2) and SIT 1166 (T1, n = 1) were found. In the XDR-TB isolates, SIT 464 (Ural, n = 1) was the only new SIT found to be evolved (Table 4).

**Association of genetic mutations in gyrA and rrs genes and phenotypic drug resistance.** Among the FQ resistant isolates, ΔWT3-D94G (40.8%) was most prevalent mutation and the frequency of this mutation was higher in Beijing strain (45.1%) followed by CAS (27.4%), X (9.8%), Unique (7.8%), T (5.8%), Manu and H (1.9%). The ΔWT2-A90V mutation (24.8%) was most common mutation in the Beijing (32.2%) and CAS (25.8%) strains. Only Beijing strain (100%) was associated with ΔWT3-D94N/Y (4.8%) mutations. However, among the AG/CP resistant isolates, ΔWT1-A1401G (57.9%) was the most prevalent mutation and the frequency of this mutation was higher (57.9%) in Beijing strains (Table 4).

**Discussion.** The first decade of this century witnessed the emergence of XDR-TB strains for which management is extremely difficult, and this imposed serious concerns for the health care systems around the world. To develop tests to diagnose XDR-MTB strains at the earliest possible time have become an urgent need. Various research organizations are working untiringly to develop such tests and devices. A German based company developed the first version of MTBDRsl for rapid screening of FQ and AG/CP resistance in the MDR-TB isolates. After preliminary evaluations, in 2013, the WHO recommended use of this test to rule out the XDR-TB. However, despite India being the high TB burden country, its usefulness of this test has not been evaluated on a large number of isolates. In the present work, we evaluated the efficiency of MTBDRsl V.1 in comparison to the second-line DST using MGIT-960 system, which still remain the gold standard for second line drug susceptibility testing. We also analysed for the first time, the association of various lineages of MTB with genetic mutational patterns. Even though it is a molecular test, which is prone to several procedural errors, we found that the test protocol is highly standardized giving no invalid results, which means that all (100%) tests were valid. Taking the phenotypic MGIT-960 system as the gold standard, we found very high (97%) concordance of MTBDRsl for detecting the second drug susceptible isolates and for FQ resistance detection (93.5%). However, more improvised version will be required to detect AG/CP resistance, where its performance was not found very high (50%). Similar findings regarding detection of AG/CP resistance have also been previously published from Spain and China reporting sensitivity of 56% and 67% respectively. Nevertheless, the test was found extremely good (94.1%) for XDR-TB detection in our study as well as by other studies published from Serbia and Netherlands both showing 100% sensitivity. Similar to our results the lower detection rate for EMB resistance of 56.2% was reported from China. These authors performed this test on MDR-TB isolates and emphasized identification of other mutations for detection of EMB resistance for improvement of the test. Hence the identification of novel mutations outside the QRDR gene region of gyrA and gyrB and rrs gene is urgently needed. The new version of the assay (i.e. MTBDRsl V.2.0) has been recently developed by the company for improvement to overall performance of MTBDRsl V.1 and in particular to its sensitivity for detection of kanamycin resistance. However, at the time of study this version was not available in India.

While correlating the association of gyrA and rrs gene mutations in various lineages we found that even though most of our isolates were from the Northern-Western part of India (270; 75.2%) where prevalence of
CAS strain is predominant yet the association of Beijing isolates was statistically significantly high (p=0.0006) with gyrA gene mutations but insignificant (p=0.079) with rrs gene mutations. Beijing strains are considerably prevalent in South East Asia and North-Eastern region of India, where its is posing a major concern due to its high prevalence among the MDR-TB patients.19,37 From these observations, recent studies from India show that this strain is spreading fast to other parts of India and neighbouring countries14, which can be warning signal to the TB control programme managers. Within the Beijing genotype SIT1 was most predominant share type amongst the pre-XDR and XDR-TB isolates in our study. SIT1 was also reported predominant among XDR-TB isolates from Africa (34%) and Russia (9.5%)37,38. We for the first time reported 18 new SITs and 5 Orphans in the Indian MDR-TB isolates, though these types have previously been reported from other countries, like SIT67 (1.4%) from United States and Mexico, SIT243 (1.1%) from Zambia, Vietnam and Italy, SIT 794 (1.1%) from Bangladesh, Pakistan and United States39–43. The two newly evolving SITs in the Beijing genotype [SIT 1168 (0.8%) and SIT 190 (0.5%)], were found only in the MDR and pre-XDR TB isolates. These SITs have been reported from Unites States, Thailand, Japan, Vietnam and China, but never from India41,42. The Ural SIT 464 was the only strain evolved among the XDR-TB isolate which have never been reported from India but only from United States of America39. Evolution of new share types among the MDR, pre-XDR and XDR-TB isolates from India indicates these strains got transmitted to India through migration of population from such geographical regions in recent years14. We also report association of Beijing genotypes with very high frequency of 2 gyrA gene mutations-ΔWT3-D94G and ΔWT2-A90V (Table 4).

In conclusion, even though our study had some limitations such as not being able to monitor the progression of disease and treatment outcome of patients due to the use of archival MDR culture isolates, our study signifies that MTBDRsl V1 is a good diagnostic tool for the detection of pre-XDR and XDR-TB. We propose that MTBDRsl V1 should be used on all MDR-TB isolates in place of phenotypic culture DST methods, till its second version is made available, in the programmatic mode. This strategy is more pertinent for countries and regions where pre-XDR and XDR-TB prevalence is high. We also conclude that more prospective genotyping studies along with next-generation sequencing methods be implemented in order to ensure the understanding of the vulnerability of some genotypes to the drug resistance development and the molecular mechanisms leading to the emergence of pre-XDR and XDR-TB strains.

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Author Contributions

Rufai S.B. performed the experiments and wrote the draft manuscript, J.S. performed genotyping, analysed the data and contributed in manuscript preparation, P.K. helped in MTBDRsl experiments, P.M. supervised the work, S.S. supervised and coordinated the work, reviewed and finalised the manuscript, and arranged reagents and chemicals.

Additional Information

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