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# Heteroresistance to rifampicin & isoniazid in clinical samples of patients with presumptive drug-resistant tuberculosis in Central India

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***Background & objectives*: A combination of resistant and susceptible *Mycobacterium tuberculosis* (MTB) isolated from clinical specimens is referred to as heteroresistance. Heteroresistance leads to difficulties in drug resistance testing and may adversely affect treatment outcomes. The present study estimated the proportion of heteroresistance among MTB in clinical samples of presumptive drug-resistant tuberculosis (TB) patients in Central India.**

***Methods*: A retrospective analysis of data generated from line probe assay (LPA) at a tertiary care hospital in Central India between January 2013 and December 2018 was carried out. A heteroresistant MTB in a sample was indicated by the presence of both wild-type and mutant-type patterns on an LPA strip.**

***Results*: Data analysis was carried out on interpretable 11,788 LPA results. Heteroresistance in MTB was detected in 637 (5.4%) samples. Of these, heteroresistance in MTB was detected in 413 (64.8%), 163 (25.5%) and 61 (9.5%) samples with respect to *rpoB, katG* and *inhA* genes, respectively.**

***Interpretation & conclusions*: Heteroresistance is considered a preliminary step in the development of drug resistance. Delayed or suboptimal anti-tubercular therapy in patients with heteroresistance of MTB may elicit full clinical resistance and negatively impact the National TB Elimination Programme. Further studies are, however, needed to determine the impact of heteroresistance on treatment outcomes in individual patients.**

**Key words** Central India - heteroresistance - presumptive drug resistance - tuberculosis

Drug resistance in *Mycobacterium tuberculosis* (MTB) is commonly associated with genes that confer resistance to a specific drug or a group of drugs. In some patients, both susceptible and resistant MTB strains are present in the clinical specimens, *i.e.* wild-type (WT) and mutant organisms coexist in the same sample or clinical isolate, which is referred to as heteroresistance[1,2]. Heteroresistance can occur from mixed infection or clonal evolution within the same strain, which can alter bacterial subpopulations[3]. If a drug-sensitive patient is treated sub-optimally or if the initiation of treatment is delayed, a small proportion

of the resistant subpopulation of the MTB strains may replicate surpassing the sensitive strains to result in full clinical resistance[4]. This adversely affects treatment outcomes[5]. Unexplained treatment failures in patients initially diagnosed as having drug-sensitive tuberculosis (TB) have been associated with heteroresistance[6]. An increment in the resistant subpopulation of MTB during treatment can transform a drug-sensitive TB patient into a drug-resistant TB patient[7]. Moreover, such patients have the potential to transmit resistant MTB subpopulation in the community. The diagnostic limitations of rapid molecular assays for the detection of heteroresistance in TB patients could also elongate the chain of transmission of resistant bacilli.

Heteroresistance is considered to be a stage that occurs before full resistance. Furthermore, the clinical relevance of heteroresistance is also not clear; however, this phenomenon does lead to difficulties not only in drug resistance testing, but also successful therapy as reported earlier[1,8]. This is because heteroresistance represents various evolutionary stages of drug resistance during treatment[1]. Moreover, a small proportion of the heteroresistant population may remain undetectable using molecular diagnosis techniques due to the presence of any mutation in the targeted region[9].

The clinical significance of heteroresistance is crucial, particularly in drug-sensitive TB patients. The detection of heteroresistance, if present, would help prevent the emergence of a resistant subpopulation of MTB in drug-sensitive TB patients. However, in general, the detection of heteroresistance is based on the bacillary load present in the clinical samples[10].

Drug-sensitive TB patients with failed treatment outcomes are eligible for line probe assay (LPA) under the National TB Elimination Programme (NTEP)[11]. LPA can detect heteroresistance to rifampicin and isoniazid in MTB directly in smear-positive pulmonary samples[12]. The simultaneous presence of WT, as well as corresponding mutation (MUT) bands in LPA indicate the presence of heteroresistance to rifampicin and/or isoniazid[13]. The present study aimed to estimate the proportion of heteroresistance in MTB by LPA in clinical samples received from presumptive drug-resistant TB patients in Central India under programmatic conditions.

## Material & Methods

A retrospective analysis of data generated using LPA between January 2013 and December 2018 was carried out at Bhopal Memorial Hospital and Research Centre, Bhopal, a Tertiary Care Hospital in Central India. The study protocol was approved by the Institutional Ethics Committee. There were 23,737 clinical samples received as a part of the mandated diagnostic workflow under the NTEP for LPA from the presumptive drug-resistant TB patients who belonged to the various districts of Madhya Pradesh.

Presumptive drug-resistant TB patients include those who are eligible for rifampicin resistance screening at the time of diagnosis of TB and/or during treatment for drug-susceptible TB or isoniazid mono/poly drug-resistant TB. These included newly detected TB cases with treatment failure, smear positive previously treated cases who remained smear positive from fourth month onwards, all pulmonary TB cases who were contacts of known multidrug-resistant TB (MDR) individuals presenting to the study site, all smear positive cases of pulmonary TB who received treatment at the time diagnosis, any smear positive cases at follow up in new or previously treated cases, all previously treated pulmonary TB cases that were smear negative at diagnosis and HIV-TB co-infected cases at diagnosis[14]. Heteroresistance was studied in these presumptive drug-resistant TB patients.

Smear microscopy was carried out and the samples found to be positive for acid-fast bacilli (AFB) were processed using the N-acetyl-l-cysteine NaOH digestion and decontamination method[15]. DNA was extracted from the processed samples using a GenoLyse kit (Hain Lifescience GmbH, Nehren, Germany). Extra-pulmonary and smear-negative pulmonary TB samples were cultured on Lowenstein–Jensen (LJ) medium after digestion and decontamination and DNA was extracted from the culture isolates. PCR and hybridization for LPA were conducted as recommended by the manufacturer. A methodology flow diagramme is shown in Figure 1.

Resistance to rifampicin and isoniazid was interpreted as per the standard protocol of the kit GenoType MTBDR*plus*, VER 2.0, Hain Lifescience GmbH, Nehren, Germany. Version 2.0 of the GenoType MTBDR kit was approved by the World Health Organization (WHO) in 2008, which included an additional gene *inhA* for the detection of low-level resistance to isoniazid[16].

The resistance detected by LPA was defined as per the guidelines of the programmatic management of drug-resistant TB[13]. A Mono resistance was defined as:

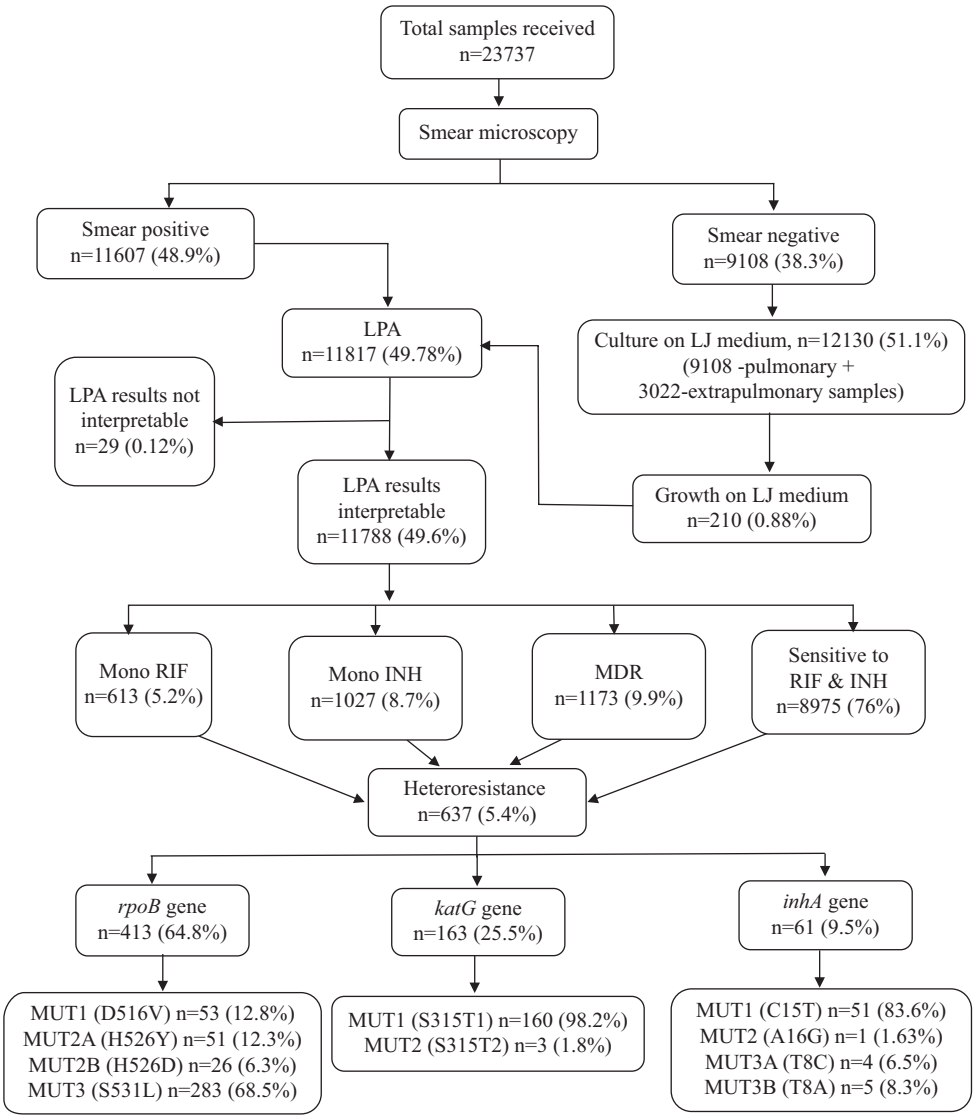


**Fig. 1.** Flow diagram of study samples. LJ medium: Löwenstein-Jensen medium; LPA: line probe assay; Mono RIF: resistance to rifampicin only; Mono INH: resistance to isoniazid only; RIF: rifampicin; INH: isoniazid

resistance only to first-line anti-TB drugs. MDR was defined as: resistance to isoniazid as well as rifampicin with/without any resistance to other first-line anti-TB drugs[14].

## Results

A total of 23,737 samples were received over a period of six years from 2013 to 2018. Of 23,737 samples, 11,607 (48.9%) were smear-positive for AFB. The remaining 12,130 (51.1%) samples [smear negative = 9108 (38.3%) and extra-pulmonary = 3022 (12.7%)] were inoculated on LJ medium. Growth in LJ media was found in 210 (0.88%) samples. LPA was carried out on 11,817 (11,607 smear-positive samples and 210 culture isolates) (49.78%) samples, 29 (0.12%) of which were not interpretable, and therefore, excluded from the analysis. Data analysis was carried out on all interpretable 11,788 (49.6%) samples. Year-wise distribution of these samples, received from presumptive drug-resistant TB patients, is shown in Table I. Of the 11,788 samples, mono resistance to rifampicin was detected in 613 (5.2%) samples and mono resistance to isoniazid was detected in 1027 (8.7%) samples. MDR, that is, resistance to both isoniazid and rifampicin, was detected in 1173 (9.9%) samples. There were 8975 (76.1%) samples with sensitivity to both rifampicin and isoniazid (Table I). Overall resistance to rifampicin (including MDR and mono resistance to rifampicin) was detected in 1786 (15.2%) samples. Overall resistance to isoniazid

**Table I.** Year-wise distribution of samples from presumptive drug-resistant tuberculosis patients and drug susceptibility pattern in *Mycobacterium tuberculosis* detected by line probe assay (n=11,788)

| Year | Sample received | Rifampicin mono resistance, n (%) | Isoniazid mono resistance, n (%) | MDR, n (%) | Rifampicin and isoniazid sensitive, n (%) |
|---|---|---|---|---|---|
| 2013 | 1540 | 171 (11.1) | 135 (8.7) | 280 (18.1) | 1034 (67.1) |
| 2014 | 1860 | 128 (6.8) | 191 (10.2) | 245 (13.1) | 1280 (68.8) |
| 2015 | 2529 | 142 (5.6) | 238 (9.4) | 274 (10.8) | 1798 (71.09) |
| 2016 | 1461 | 67 (4.5) | 130 (8.8) | 144 (9.8) | 1150 (78.7) |
| 2017 | 974 | 57 (5.8) | 76 (7.8) | 89 (9.1) | 732 (75.1) |
| 2018 | 3424 | 48 (1.4) | 257 (7.5) | 141 (4.1) | 2981 (87.06) |
| Total | 11,788 | 613 (5.2) | 1027 (8.7) | 1173 (9.9) | 8975 (76) |

MDR, multidrug resistance

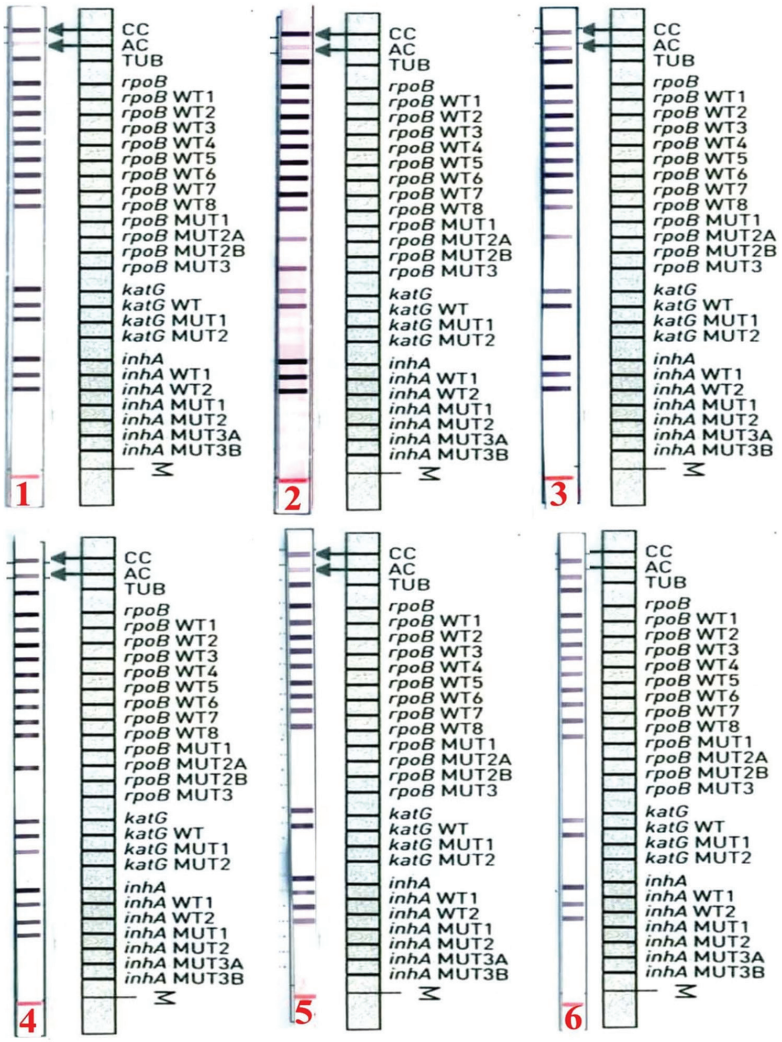


**Fig. 2.** LPA strips shown here are representative of the years 2014 (strip 5), 2015 (strip 4), 2016 (strips 1 and 2), 2017 (strip 3) and 2018 (strip 6). The LPA strips 1 to 5 show the simultaneous presence of all WT as well as MUT band/s indicating heteroresistance in MTB strains. LPA strip 1 shows heteroresistance to *katG* gene, LPA strip 2 shows heteroresistance to the *rpoB* gene, LPA strip 4 shows heteroresistance to all three genes-*rpoB, katG* and *inhA* gene and LPA strip 5 shows heteroresistance to *inhA* gene. LPA strip 6 shows a sensitive band pattern of an MTB strain with the presence of all WT but no MUT band. LPA: line probe assay; WT: wild-type; MUT: mutation; MTB: *Mycobacterium tuberculosis*; CC: conjugate control; AC: amplification control; TUB: band specific to tuberculosis species.

(including MDR and mono resistance to isoniazid) was detected in 2200 (18.7%) samples.

The above-mentioned samples in which resistance to rifampicin and/or isoniazid in MTB was detected, also included those in which heteroresistance was detected. Hybridization with all WT as well as with one or more mutant probes in an LPA strip indicated the presence of heteroresistance (Fig. 2).

Of the 11,788 samples from presumptive drug-resistant TB patients, heteroresistance in MTB was detected in 5.4 per cent (637/11,788) samples. Furthermore, these included MTB heteroresistance with respect to the *rpoB* gene in 3.5 per cent (413/11,788) samples, *katG* gene in 1.38 per cent (163/11,788) samples and *inhA* gene in 0.51 per cent (61/11,788) samples.

Of the 1786 samples with rifampicin resistance, the proportion of heteroresistance with respect to the *rpoB* gene was seen in 23.2 per cent (413/1786). Of the 2200 samples with isoniazid resistance, the proportion of heteroresistance with respect to the *katG* gene was seen in 7.4 per cent (163/2200) and *inhA* gene was 2.8 per cent (61/2200). Among the 637 samples with heteroresistant MTB strains, MDR was detected in 332 (52%) samples, monoresistance to rifampicin was detected in 211 (33%) samples and monoresistance to isoniazid was detected in 94 (15%) samples.

Heteroresistance in the *rpoB* gene, *KatG* gene and *inhA* gene in MTB strains over a period of six years is shown in Table II. Of the 637 MTB heteroresistant strains, 413 (64.8%) exhibited heteroresistance with respect to the *rpoB* gene with the presence of all eight WT bands along with one or more MUT bands [MUT1(D516V), MUT2A(H526Y), MUT2B(H526D) and MUT3(S531L)]. The most frequent mutation was observed in MUT3(S531L) (283/413, 68.5%) codon, followed by mutations in MUT1(D516V) (53/413, 12.8%), MUT2A(H526Y) (51/413, 12.3%) and MUT2B(H526D) (26/413, 6.3%) codons.

Of the 637 MTB heteroresistant strains, 163 (25.5%) exhibited heteroresistance with respect to the *katG* gene with the presence of a WT band along with the MUT band [MUT1(S315T1) or MUT2(S315T2)]. The most frequent mutation was observed in MUT1 (S315T1) (160/161, 98.2%) codon, followed by mutation in MUT2(S315T2) (3/161, 1.8%) codon (Table III).

Furthermore, of the 637 MTB heteroresistant strains, 61 (9.5%) exhibited heteroresistance with respect to the *inhA* gene with the presence of both WT bands along with one or more MUT bands [MUT1(C15T), MUT2(A16G), MUT3A(T8C) and MUT3B(T8A)]. The most frequent mutation was observed in MUT1(C15T) (51/61, 83.6%) codon, followed by mutations in MUT3B(T8A) (5/61, 8.3%), MUT3A(T8C) (4/61, 6.5%) and MUT2 (A16G) (1/61, 1.63%) codons (Table III).

**Table II.** Heteroresistance in *rpoB*, *KatG* and *inhA* genes in *Mycobacterium tuberculosis* over the period of six years

| Year | Number of samples | Heteroresistance | | |
|---|---|---|---|---|
| | | *rpoB* gene, n (%) | *KatG* gene, n (%) | *inhA* gene, n (%) |
| 2013 | 1540 | 162 (10.5) | 25 (1.6) | 9 (0.5) |
| 2014 | 1860 | 92 (4.9) | 22 (1.18) | 8 (0.4) |
| 2015 | 2529 | 72 (2.8) | 33 (1.3) | 9 (0.3) |
| 2016 | 1461 | 20 (1.3) | 15 (1.02) | 8 (0.5) |
| 2017 | 974 | 10 (1.02) | 14 (1.43) | 1 (0.1) |
| 2018 | 3424 | 57 (1.6) | 54 (1.5) | 26 (0.7) |
| Total | 11,788 | 413 (3.5) | 163 (1.38) | 61 (0.51) |

## Discussion

Accurate laboratory diagnosis of drug-resistant TB is a necessity for appropriate treatment in TB patients. However, in the case of heteroresistance, the quantum of resistant strains in the clinical sample would determine the diagnosis of drug resistance/drug sensitivity. GeneXpert (CBNAAT) can detect rifampicin resistance only when >50 per cent of MTB strains in the samples are resistant, and LPA can detect rifampicin resistance when ≥5 per cent of MTB strains in the samples are resistant[10]. The presence of undetectable resistant TB bacilli in the clinical samples may account for the unexplained treatment failures in TB patients.

The heteroresistant cases which are missed by the routine molecular diagnosis may respond well to the anti-TB therapy; however, immunosuppression, non-compliance and delayed or suboptimal treatment are largely responsible for reverting the case[17]. Undiagnosed heteroresistance may have long-term impacts on the programme and the affected patients. If a patient with undiagnosed heteroresistance is on a drug-sensitive TB regimen, it is likely that in the presence of antibiotic pressure, resistant strains may overgrow, leading to a worse treatment outcome[18]. Furthermore, TB patients may relapse due to heteroresistance, even after the successful completion of the anti-TB regimen[19].

**Table III.** Frequency distribution of mutations in heteroresistant strains of *Mycobacterium tuberculosis*

| Gene | MUT | n (%) |
|---|---|---|
| *rpoB* gene (n=413) | MUT1 (D516V) | 53 (12.8) |
| | MUT2A (H526Y) | 51 (12.3) |
| | MUT2B (H526D) | 26 (6.3) |
| | MUT3 (S531L) | 283 (68.5) |
| *katG* gene (n=163) | MUT1 (S315T1) | 160 (98.2) |
| | MUT2 (S315T2) | 3 (1.8) |
| *inhA* gene (n=61) | MUT1 (C15T) | 51 (83.6) |
| | MUT2 (A16G) | 1 (1.63) |
| | MUT3A (T8C) | 4 (6.5) |
| | MUT3B (T8A) | 5 (8.3) |
| Total | | 637 |

MUT, mutation

Therefore, early detection of heteroresistance may help avoid the selection of resistant strains during anti-TB treatment and ensure successful treatment outcomes under the national programme [20].

The heteroresistance in MTB has been evaluated in different geographic areas[21-23], but so far there are no data from Central India. Few studies from central India, however, have reported on drug-resistant TB[24,25]. A study from Central India while studying the situation of drug-resistant TB among the Saharia tribe reported a high prevalence of 2.2 per cent MDR among new and 8.8 per cent MDR among previously treated TB patients[25]. In contrast, MDR was reported to be slightly lower than that reported in our study (9.9%). Another study[26] from the same region reported 18.1 per cent isoniazid resistance and 4.5 per cent MDR among the treatment defaulters of the Saharia tribe, which is similar to the isoniazid resistance observed in our study (18.7%). Another study[27] from Central India has reported 61.2 per cent drug-resistant TB cases while studying the factors related to TB patients visiting a regional DR-TB centre. Although the study, did not differentiate between the type of DR TB cases[27].

In the present study, among all presumptive drug-resistant TB cases (n=11,788), 3.5 per cent heteroresistance cases could be detected by LPA with respect to the *rpoB* gene, which confers resistance to rifampicin. The proportion of heteroresistance with respect to the *katG* (1.4%) and *inhA* (0.5%) genes, which confer resistance to isoniazid, was low. We also identified 58 MDR strains that simultaneously showed heteroresistance in the *rpoB* gene as well in the *katG* and/or *inhA* genes.

The proportion of heteroresistance was found to vary geographically. A study from south India has reported a heteroresistance of 29.3 per cent with respect to the *rpoB* gene, 22.2 per cent for *katG* gene and 11.2 per cent for *inhA* gene in drug-resistant TB cases[28]. In contrast to this study, we identified a lower proportion of heteroresistance in the *rpoB* gene (23.1%), *katG* gene (7.4%) and *inhA* gene (2.8%) among drug-resistant TB cases. A study from Delhi, India, reported 38.8 per cent heteroresistance with respect to the *rpoB* gene, which is higher than that of our study[29]. Another study from Delhi detected a much lower proportion of heteroresistance to isoniazid (4%) and rifampicin (1%)[30]. A study from northern India reported only 0.8 per cent heteroresistance to isoniazid by LPA[31].

A variation in the occurrence of heteroresistance is also reported outside India. A study from Italy detected 11.1 per cent heteroresistance in MTB with respect to the *katG* gene[32]. A study from the South American region observed 4.4 per cent heteroresistance in MTB isolates with respect to the *rpoB* and *katG* genes[33]. Daum *et al*[34] used next-generation sequencing to characterize MDR and extensively drug-resistant clinical isolates of MTB collected from Ukraine and the southern Crimean Peninsula and identified heteroresistance in 7.2 per cent MTB isolates with respect to the *rpoB* gene. A study from China detected three (3%) rifampicin heteroresistant strains out of 99 culture isolates from newly diagnosed sputum smear-positive TB patients[35]. In Ethiopia, 4.7 per cent heteroresistance to rifampicin and 1.13 per cent heteroresistance to isoniazid has been reported[22] which were almost similar to our study. A 20 per cent of heteroresistance to rifampin and/or isoniazid was found in Tashkent, Uzbekistan which is considerably higher than that of the overall heteroresistance found in our study (5.4%)[19].

Heteroresistance is an unstable phenomenon which occurs mainly due to the tandem amplification of target genes[18]. The uncertainty in the expression of drug-resistant genes may be a plausible explanation for variations in the frequencies of heteroresistance in regional and global populations.

Heteroresistance, a phenomenon in which two different susceptibility patterns are identified, has been found to be associated with all the target genes of LPA

that are *rpoB*, *katG* and *inhA*, however, with different frequencies. As evident by our study, the *RpoB* gene (3.5%) was primarily associated with heteroresistance whereas the *inhA* gene was associated the least (0.5%) among the drug-resistant TB suspects.

The most frequent mutation in the *rpoB* gene at codon S531L is well known and has been documented widely[36-39]. With respect to the heteroresistant subpopulation of MTB, we also found the most frequent mutation at codon S531L (68.5%), followed by mutation at codon D516V (12.8%) similar to the findings of a study from north India[34]. Another study from the southern coastal region of Andhra Pradesh, India, reported the most prominent mutation at codon S531L of the *rpoB* gene, however, the mutation at codon D516V was found least frequently, which is in contrast to our findings[16].

Mutations at codon S315T1 of the *katG* gene and C15T of the *inhA* gene are the most dominant mutations which were depicted in our study. The predominance of mutation at codon S315T1 of the *katG* gene and C15T of the *inhA* gene have also been reported from Punjab and Andhra Pradesh in heteroresistant MTB subpopulations[16,40]. Gupta *et al*[26] reported S315T1 and S531L mutations in *katG* and *rpoB* genes, respectively in 3.8 per cent of MDR isolates.

Heteroresistance is often present as disputed mutations, as they do not replace the WT population as rapidly as high-level mutations do[41]. Most of the disputed mutations are not identified by LPA with the presence of MUT bands, which mainly target frequent high-level mutations. Disputed heteroresistance may present as a weak WT band, without the presence of a MUT band[42]. This may impair the detection of heteroresistance in some instances. It is also likely that undetected heteroresistance among the patients diagnosed as having drug-sensitive TB turn out with poor treatment outcomes or treatment failures[43]. The TB patients receiving first-line antitubercular therapy but with poor treatment outcomes are typically categorized under presumptive drug-resistant TB patients. The sputa of such patients are usually sent to the reference laboratories for detection of drug resistance to rifampicin and isoniazid by LPA.

We analyzed the treatment outcomes of the patients who were diagnosed as having drug-sensitive TB between the years 2017 and 2018 in the State of Madhya Pradesh. The data of these patients are available in the NI-KSHAY portal[44]. In 2017, of 130,449 patients to whom treatment outcomes were assigned, 4010 (3.0%) died, and 1220 (0.9%) failed the standard treatment. In 2018, of 148,120 patients to whom treatment outcomes were assigned, 4316 (2.9%) died and 1054 (0.7%) failed the standard treatment[44]. Although in this study, we could not find the treatment outcomes for individual patients in whom heteroresistant MTB populations were detected, it is possible that poor treatment outcomes may be related, to some extent, to heteroresistance[10], since it is considered to be a preliminary step in the development of drug resistance[19]. We were unable to collect information on the type of presumptive drug resistance in TB patients either. This is a retrospective study; therefore, information on a real-time basis could not be possible on these patients. Further follow up investigations are needed to strengthen the findings reported in this study. Given the current incident rate of treatment failures, prospective targeted studies are required from different geographical locations of Central India with more classified data and a larger sample size that could help determine the most informative and actual scenario of the heteroresistance in MTB. As the study is retrospective, certain drawbacks/limitations have remained that essentially suggest further follow up investigations are needed to strengthen the findings reported here.

The major limitation of the study was that we could not do the whole genome sequencing of the MTB subpopulation identified as having heteroresistance since no separate funding was received for this study. Therefore, confirmation of true heteroresistance and the number of samples in which detection of heteroresistant MTB subpopulation was missed, could not be done. Further, culture and drug susceptibility was not carried out on the samples received for LPA under the programme. Therefore, the phenotypic sensitivity of heteroresistant strains was not available for further analysis.

In conclusion, heteroresistance in MTB was detected in 5.4 per cent of samples received from the presumptive drug-resistant TB patients, which is a significant proportion in terms of absolute numbers. It is possible that treatment outcomes may not be optimal in some drug-sensitive TB patients in whom heteroresistance remained undiagnosed. The 5.4 per cent of patients in whom we detected heteroresistance in MTB may be associated with such cases. These patients were receiving first-line antitubercular regimens, and are now classified as presumptive drug-resistant TB patients due to the failed treatment outcomes. Heteroresistance may be missed by the currently

available laboratory technologies utilized through the NTEP. It may be worthwhile to explore avenues for making available whole genome sequencing for the population who needs it. Further, studies are needed to examine the impact of heteroresistance in individual patients on treatment outcomes. Algorithms could then be developed to diagnose heteroresistance and address the related outcomes.

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***Conflicts of Interest*:** None.

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