Effective Pragmatic Approach of Diagnosis of Multidrug-Resistant Tuberculosis by High-Resolution Melt Curve Assay

Sanjay Singh Negi¹, Priyanka Singh¹, Anudita Bhargava¹, Sachin Chandrakar², Ujjwala Gaikwad¹, Padma Das¹, Ajoy Behra²
Departments of ¹Microbiology and ²Pulmonary Medicine, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India

Abstract

Background: Effective management of multidrug-resistant tuberculosis (MDR-TB) requires cost-effective and rapid screening of rifampicin (RIF) and isoniazid (INH) resistance. Accordingly, a highly promising high-resolution melting (HRM) analysis was evaluated in the detection of mutation in \textit{rpoB}, \textit{katG} gene and \textit{inhA} promoter region in \textit{Mycobacterium tuberculosis} isolates. Methods: A total of 143 \textit{M. tuberculosis} isolates comprising phenotypically confirmed 94 MDR and 49 sensitive isolates were analyzed by HRM following real-time-polymerase chain reaction in comparison to gold standard of targeted DNA sequencing of \textit{rpoB}, \textit{katG} gene and \textit{inhA} promoter region. Results: HRM correctly identified MDR-TB by rapid and accurate detection of predominantly and infrequently occurring specific single nucleotide polymorphism in \textit{rpoB}, \textit{katG} gene and \textit{inhA} promoter region. \textit{rpoB} HRM showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 98% each respectively. Predominantly, S531 L/W (TCG → TTG/TGG) mutation accounted for 68.47% of RIF resistance followed by H526Y/R (13.04%, CAC → TAC/CGC), D516Y/V/G (10.86%, GAC → TAC/GTC/GGC), Q513P (4.34%, CAA → CCA), and one rare mutation at codon position L533A (CTG → CGG). Combined \textit{KatG} and \textit{inhA} HRM sensitivity, specificity, PPV, and NPV were 90%, 100%, 100%, and 84.48% respectively and detected frequent mutation at codon position S315T/I/N (70%, AGC → ACC, AGC → ACT, AGC → AAC) and rare mutation at codon position T314P (3.3%, ACC → CCC) and 329 (2.2%, GAC → GCC) of \textit{katG} gene. In \textit{inhA}, mutations were recorded at mostly promoter position −15 (10%, C → T) and infrequently at −8 (3.3%, T → G, T → C). HRM assay limitation noticed in recognizing silent mutation in \textit{rpoB} as a mutant, nondetection of infrequent mutation S310A in \textit{katG}, and the inability of detecting mutation outside the targeted region of investigated genes. Conclusion: HRM may prove to be a vital molecular assay in rapid screening of TB cases for early detection of MDR TB, leading to early evidenced-based initiation of antitubercular treatment that will significantly reduce MDR transmission.

Keywords: High-resolution melting, \textit{inhA}, \textit{katG}, multidrug-resistant tuberculosis, \textit{rpoB}

INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB) representing \textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}) strain exhibiting resistance to at least isoniazid (INH) and rifampicin (RIF) has posed a serious threat to the effective management of TB due to longer treatment requirement with a high probability of poor adherence, potential transmission, and chances to turning into more fearsome form of extensively drug-resistant (XDR) TB.¹² The World Health Organization (WHO) in the global TB report, 2017, estimated at least 600,000 MDR-TB cases worldwide, of which India alone constitutes 25% (147000).³⁴ Various studies have shown concern over the emergence of MDR and XDR-TB in new and previously treated cases.¹³⁻⁶ First national anti-TB drug resistance survey 2014–16 report from Government of India showed 6.19% (confidence interval [CI]):

Address for correspondence: Dr. Sanjay Singh Negi, Department of Microbiology, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India. E-mail: negidr@yahoo.co.in

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5.54%–6.90%) MDR among all TB patients with 2.84% (CI 2.27%–3.50%) new and 11.60% (CI 10.21%–13.15%) among previously treated TB patients. In Chhattisgarh, MDR-TB was reported as 4.26% in new TB patients and 6.06% in previously treated patients.[15] It is a major threat to the vision of WHO and Government of India to eliminate TB in India by 2025.

Effective, rapid diagnosis of MDR-TB is of the paramount requirement of the hour to provide the early sensitivity pattern of M. tuberculosis to the clinician to initiate early evidence-based effective treatment to circumvent its secondary transmission.

Conventional phenotypic drug susceptibility test using 1% proportion, absolute concentration, and resistance ratio methods using either solid media such as Lowenstein-Jensen (LJ) and Middlebrook 7H10/11 or liquid automated system such as BACTEC Mycobacteria growth indicator tube (MGIT) 960 (BD, Sparks, MD, USA) and BacT/ALERT (Biomerieux, USA) have their own limitations of providing result in 3–6 weeks. Due to rapid advancement in understanding the molecular basis of resistance to RIF and INH, several molecular methods developed in the past decade or so have come up with good sensitivity and specificity. Commercial assay such as GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), two commercially available reverse hybridization-based line probe assay of INNO-LiPA RIF (Innogenetics, NV, Ghent, Belgium) for RIF resistance detection, and genotype MTB DR plus/MDRDrsl (HAIN Lifescience, Nehren, Germany) for simultaneous detection of both RIF and INH resistance and pyrosequencing provides rapid molecular diagnosis of MDR-TB.[16] However, these tests too suffer with limitations of high cost, complex, labor-intensive, specialized infrastructure requirement, and targeting specific frequent mutation due to which infrequent or rare mutation may be missed leading to false negative reporting in otherwise true MDR cases. These limitations adequately justify the need of developing simple, cheap, and rapid diagnostic assay to detect drug resistance mutations.

Addressing these issues, high-resolution melting (HRM) curve analysis assay is relatively a simple, rapid, and inexpensive polymerase chain reaction (PCR)-based closed tube assay for detection of single nucleotide polymorphism (SNP) by demonstrating the fluorescence changes in the melting temperature of amplified product. HRM assay can be utilized without the need of specific probes for the detection of frequent, rare, and novel mutation in a large number of samples in a short time.

Accordingly, in the present study, we have evaluated HRM assay to rapidly detect MDR-TB by detecting SNP in rpoB, katG gene and the inhA promoter region which had been verified and characterized by targeted sequencing.

**METHODS**

The study was performed in two centers with conventional drug sensitivity test (DST) being performed at Intermediate Reference Laboratory (IRL) of Revised National Tuberculosis Control Program, Lalpur, Raipur, Chhattisgarh, by 1% proportion method using MGIT 960 according to the manufacturer’s instructions and molecular studies being done at Molecular Diagnostic Laboratory of Microbiology Department, AIIMS, Raipur, Chhattisgarh by HRM and DNA sequencing to rapidly detect MDR-TB.[12-14]

**Mycobacterial isolates**

A total of 143 mycobacterial isolates comprising phenotypically proven 94 MDR and 49 primary antitubercular drug (INH, RMP, ethambutol, and streptomycin) susceptible clinical isolates were randomly provided by IRL in MGIT broth. All the samples were subcultured on LJ medium to ascertain the growth of M. tuberculosis as per standard laboratory protocol. The laboratory reference strain M. tuberculosis H37Rv was used as the wild-type control. The study was approved by the Institute Ethics and Research Committee of AIIMS, Raipur.

**DNA Extraction**

DNA was extracted by manual heating and purification. Briefly, the 0.5 ml of liquid broth of every sample was centrifuged at 12,000 rpm to obtain the deposit. Deposit was suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM ethylenediaminetetraacetic acid) in sterile 1.5 ml Eppendorf tube. The tubes were vortexed for around 10 s before heating in dry bath at 99°C for 20 min. Then, the sample was centrifuged at 15,000 rpm for 10 min. The DNA in the upper layer was concentrated and further purified by washing with AW1 and AW2 wash buffer provided in the commercial QIAmp DNA Mini Kit (QIAGEN, Germany) according to the kit instructions. The genomic DNA was eluted in 100 µl TE buffer and stored at −20°C until its further use.

**High-resolution melting assay following real-time polymerase chain reaction**

Eva Green dye which was a part of precision melt supermix (Bio-Rad) is a saturating DNA binding dye producing an amplicon-specific melting curve detecting any presence of DNA sequence variation. The DNA from each isolate was amplified by PCR using rpoB, katG gene and inhA promoter primers as described in Table 1. Each of the reaction consists of >10 supermix, 10 picomole forward and reverse primer, and 3 µl of DNA in a final volume of 20 µl per reaction. The thermal cycling parameter was the activation of enzyme at 98°C for 2 min and 40 cycles at 98°C for 10 s and 62°C for 30 s (for katG 63°C for 30 s). In rpoB HRM, PCR amplification cycle was followed with 1 min at 95°C and 70°C for 1 min. katG and inhA PCR thermal cycle was directly subjected to melt curve. Melt curve consisted of holding step of 10 s at 70°C for rpoB and 60°C for 10 s in katG and inhA HRM followed by slow melt increase at rate of 0.2°C/s to 95°C with continuous fluorescence detection. The HRM analysis of melt curves was performed using the Precision Melt Analysis software of Bio-Rad.

**High-resolution melting interpretation**

Any mycobacterial isolate showing differences in the fluorescent melt curve from wild-type mycobacterial strain H37Rv in the normalized and temperature-shifted melting curves scale was considered as a resistant mutant. Any
isolate showing melt curve similar to H37Rv was labeled as sensitive (Wild Type).

**DNA sequencing**
It was used to confirm resistance in all phenotypically resistant isolates. Conventional PCR was performed to amplify the target sequence of rpoB, katG, and inhA using the primers listed in Table 1. The thermal cycling parameter used for amplification of 278 bp of rpoB and 231 bp of inhA included an initial denaturation at 95°C for 5 min and 35 cycles of 20 s at 95°C, 30 s at 55°C, and 30 s at 72°C with a final extension at 72°C for 5 min. The thermal cycling for amplification of 200 bp katG included initial denaturation at 95°C for 5 min, 30 cycle of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s and final elongation at 72°C for 7 min. The presence of PCR amplicon was confirmed by its specific band in 1.5% agarose gel in 1X TBE. The amplified product was purified with HiMedia PCR purification kit and used as template for sequencing PCR in a big dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The separate reaction mixture for rpoB, katG, and inhA included purified DNA (15–45 ng), 3.2 pmol of forward primer, and 4 µl of the terminator ready reaction mix supplied in the kit. The cycling parameter for sequencing included 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. The sequencing products were further purified by ethanol precipitation. The pellet was rehydrated in 15 µl of formamide and denatured at 95°C for 5 min followed by immediate ice exposure for 5 min and then loaded in ABI Prism 3130 sequencer.

**Statistical analysis**
Sensitivity, specificity, and positive and negative predictive value (PPV and NPV) of the HRM were calculated against the gold standard of sequencing result using the SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA).

**RESULTS**
A total of 143 mycobacterial isolates comprising phenotypically confirmed 94 MDR isolates and 49 sensitive isolates were analyzed by HRM curve analysis for the detection of any SNP-associated drug resistance against RIF and INH. All the isolates were also processed by DNA sequencing to ascertain exact mutational nucleotide changes and codon position to verify HRM results. All the isolates were confirmed of M. tuberculosis by amplifying 123 bp region of IS6110 and 240 bp region of mbp64 gene specific for M. tuberculosis isolates as described earlier (data not shown). Due to higher sensitivity in smaller nucleotide segment, HRM specifically targeted 133 bp region of rpoB containing 81 bp RIF resistance determining region (RRDR), 120 bp of katG, and 75 bp of inhA promoter region. The unique HRM fluorescent pattern of wild-type and mutant isolates were shown in Figures 1-3 in normalized graph mode. The baseline was represented by M. tuberculosis H37Rv. Melting temperature change started occurring between test isolate and control H37Rv and susceptible isolate between a temperature of 85 and 90°C for rpoB and 82°C–86°C for katG and 81°C–85°C for inhA.

**Detection of rifampicin resistance using high-resolution melting and sequencing**
In rpoB targeted region, HRM result successfully showed eight different cluster formation for ten different point mutations involving most frequent mutation at codon position 531, 526, and 516 followed by mutation at codon position 533, 530, and 513 [Figure 1 and Table 2]. All the cluster formations were clearly differentiated and indicated SNP in comparison to M. tuberculosis standard strain of H37Rv. HRM result was found in complete concordance with sequencing except one isolate which showed mutation at codon position 490 which was found outside the RRDR region and not included in HRM [Table 2]. According to HRM and sequencing results, maximum RIF resistance in 63 cases (68.47%) was found due to point mutation in codon position 531 with 61 isolates (64.89%) showed TCG (S)→TTC (L) and 2 isolates (2.12%) with TCG (S)→TGG (W) mutation. This was followed with codon position 533, 530, and 516 followed by mutation at codon position 533, 513, and 516.

| Table 1: Primers used in polymerase chain reaction-high resolution melting and sequencing of rpoB, katG, and inhA region of Mycobacterium tuberculosis |
|---|---|---|---|
| Primer | Target Gene | Sequence | Used for |
| rpoB F - GCACGCGATCAAGGAGTTCTTC | rpoB | HRM |
| rpoB R - CGGCACGTGCTGACAGAC | |
| rpoB F - 2F - CAAGACGTGAGGAGGCATAC | |
| rpoB R - SR - GAGCCGATCAGACGATGTTGG | |
| KatG F - 5' - AGCTCTGATGGCACCAGGAAC | KatG | Sequencing |
| KatG R - 5' - AACCGGTTCGGGGATGGTTG | |
| inhA F - 5'TCACACCCGCAACACGTACGAGC | inhA | HRM |
| inhA R - 5 ’-AGCCAGGGCTGTCGAGTCGAA | |

HRM: High-resolution melting, TB: Tuberculosis, INH: Isoniazid, RIF: Rifampicin
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mutation and 4 isolates (4.25%) with CAC (H)→CGC (R) mutation. Third most frequent mutation was found at codon position 516 in ten MDR isolates (10.6%). Among these isolates, six isolates (6.38%) showed GAC (D)→TAC (Y), three isolates (3.19%) showed GAC (D)→GTC (V), and one (1.06%) were found having GAC (D)→GGC (G) mutation. Four MDR isolates (2.12%) exhibited point mutation at codon position 513 CAA (Q)→CCA (P). A rare mutation was found at codon position 533 (CTG [L]→CGG [R]). One HRM mutant isolate was found with silent mutation at codon position 530 (CTG [L]→CTC [L]). Two phenotypic RIF-R isolates were found genotypically sensitive by HRM.
and sequencing. All 49 susceptible isolates were found with similar curve pattern in comparison to H37Rv and further verified by sequencing. The calculated sensitivity, specificity, PPV, and NPV of rpoB HRM was recorded as 98.90% (95% CI: 94.03–99.97), each respectively.

**Detection of isoniazid resistance using high-resolution melting analysis**

Combined HRM analysis of 120 bp region of katG and 75 bp inhA promoter region showed concordance between HRM and sequencing in a total of 85 isolates out of 94 [Table 3 and Figures 2 and 3]. HRM failed to detect infrequent mutations in a total of 9 isolates at various codons namely katG codon position 310 (03 isolates, AGC → GCC), 299 (05 isolates, GGC → AGC), and 341 (01 isolate, 1.08%), leaving it with a sensitivity, specificity, PPV, and NPV of 90%, 100%, 100%, and 84.48%, respectively. Among these 85 isolates, 81 were found INH resistant and 4 INH sensitive by both HRM and sequencing results. These four INH-sensitive isolates included two mono RIF-resistant isolates as confirmed by sequencing. Among 81 INH-R, 66 isolates (73.33%) showed three different point mutations at codon position 315 which included 59 isolates with AGC → ACC (56 single mutation

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**Table 2: HRM and Sequencing result of rpoB for 94 phenotypically resistant MDR and 49 susceptible M.tuberculosis isolates. Symbol used: NA (Not applicable), S (Serine), L (Leucine), W (Tryptophan), H (Histidine), Y (Tyrosine), R (Arginine), D (Aspartic Acid), V (Valine), G (Glycine), Q (Glutamine), P (Proline), A (Alanine).**

| Anti-Tuberculosis Drug | 1% proportion | Number (Mutant codon) | Nucleotide change | Percentage | Cluster | Result |
|------------------------|---------------|------------------------|-------------------|------------|---------|--------|
| Rifampicin             | RIF-S (01)    | 01(NA)                 | H37Rv             | Wild Type  | NA      | 01     | Wild Type |
|                        | RIF-R (92)    | 63 (68.47%)            | 61 isolate (S531L) | TCG (Ser) → TTG (Leu) | 64.30%   | 2      | Mutant (Resistant) |
|                        |               | 2 isolate (S531W)      | TCG (Ser) → TGG (Trp) | 2.17%     | 6       | Mutant (Resistant) |
|                        |               | 12 (13.04%)            | 8 isolate (HS26Y)  | CAC (His) → TAC (Tyr) | 8.69%    | 4      | Mutant (Resistant) |
|                        |               | 4 isolate (HS26R)      | CAC (His) → CGC (Arg) | 4.34%     | 8       | Mutant (Resistant) |
|                        |               | 10 (10.86%)            | 6 isolate (DS156Y) | GAC (Asp) → TAC (Tyr) | 6.52%    | 7      | Mutant (Resistant) |
|                        |               | 3 isolate (DS156V)     | GAC (Asp) → GTC (Val) | 3.26%     | 5       | Mutant (Resistant) |
|                        |               | 1 isolate (DS156G)     | GAC (Asp) → GCC (Gly) | 1.08%     | 8       | Mutant (Resistant) |
|                        |               | 4 (4.34%)              | (Q513P)           | CAA (Gln) → CCA (Pro) | 4.34%    | 9       | Mutant (Resistant) |
|                        |               | 1 (1.08%)              | (L530L)           | CTG (Leu) → CTC (Leu) | 1.08%    | 6       | Mutant (Resistant) |
|                        |               | 1 (1.08%)              | (L533A)           | CTG (Leu) → CGG (Arg) | 1.08%    | 3       | Mutant (Resistant) |
|                        |               | 01 (1.08%)             | Q490A             | CAG (Glu) → CGG (Arg) | 1.08%    | 1       | Wild Type |
| Rifampicin             | RIF-R (02)    | 2 (2.1%)               | Wild Type         | Wild Type   | Wild Type |
| MDR-S (49)             |               | 49 (Wild Type)         |                 |            |         |

HRM: High-resolution melting, MDR: Multidrug-resistant, RIF: Rifampicin

**Table 3: HRM and Sequencing result of katG and inhA gene for 94 phenotypically resistant MDR mycobacterial isolates. Amino acid single letter symbol involved T (Threonine), I (Isoleucine), N (Asparagine), P (Proline), D (Aspartic Acid), A (Alanine), S (Serine), G (Glycine).**

| Anti-TB drug | 1% proportion | No | Gene | Number (%) | (Mutant codon/position) | Nucleotide change | Percentage | Cluster | Result |
|--------------|---------------|----|------|------------|-------------------------|-------------------|------------|---------|--------|
| INH          | INH-R (94)    | 77 | KatG | 56 (70.0%) | S315T/I/N               | AGC (Ser) → ACC (Thr) | 1          | Mutant (Resistant) |
|              |               | 01 | (1.08%) | 06 (6.52%) |                         | AGC (Ser) → ATC (Iso) | 6          | Mutant (Resistant) |
|              |               | 03 (3.26%) | T314P | GAC (Asp) → GCC (Gly) | 1.08%     | 3        | Mutant (Resistant) |
|              |               | 02 (2.46%) | D329A | ACC (Thr) → CCC (Pro) | 1.08%     | 4        | Mutant (Resistant) |
|              |               | 03 (3.26%) | S311A | GAC (Asp) → GCC (Gly) | 1.08%     | 5        | Mutant (Resistant) |
|              |               | 05 (6.49%) | G299S | ACC (Ser) → ACC (Thr) | 1.08%     | 5        | Mutant (Resistant) |
|              |               | 01 (1.08%) | N341G | TGG (Trp) → TGG (Gly) | 1.08%     | 5        | Mutant (Resistant) |
|              |               | 10 | inhA | 09 (9.57%) | -15                    | C → T          | 2          | Mutant (Resistant) |
|              |               | 01 (1.23%) | -8    | T → G       | 2          | 3        | Mutant (Resistant) |
|              |               | 03 | KatG, inhA | 03 (3.26%) | 315 (katG) | AGC (Ser) → ACC (Thr) | 1        | Mutant (Resistant) |
|              |               | 04 | KatG, inhA | 03 (3.26%) | inhA(8)    | T → C          | 4          | Mutant (Resistant) |
|              |               | 04 | KatG, inhA | 03 (3.26%) | inhA(8)    | T → C          | 5          | Mutant (Resistant) |

HRM: High-resolution melting, TB: Tuberculosis, MDR: Multidrug resistant, INH: Isoniazid
and 3 dual mutation along with mutation at \textit{inhA} promoter codon 8), followed by 1 isolate with AGC → ACT and 6 isolates with AGC → AAC mutation. Other mutation detected in HRM was at codon position 314 (03 isolates, ACC → CCC) and 329 (02 isolates, GAC → GCC). In \textit{inhA} HRM, mutations were observed in promoter region – 15 (C → T) (9 isolates, 9.57%) and – 8 (C → T), (3 isolates had concomitant mutation at katG 315 and \textit{rpoB} 531 codon position) and – 8 (T → G) (1 isolate, 1.23%). Combined HRM of targeted regions of \textit{rpoB}, katG, and \textit{inhA} showed sensitivity and specificity of 90% and 98%, respectively.

\textbf{Turnaround time and cost}

HRM of each of the studied gene of \textit{rpoB}, katG, and \textit{inhA} required 3.30 h for completion including isolation of DNA. HRM cost per sample was found to be approximately USD 1.02 in comparison to USD 4.74 incurred in sequencing per isolates.

\textbf{Discussion}

This study evaluated the HRM assay for the detection of SNP-associated frequently dominant and rare mutations occurring in \textit{rpoB}, katG, and \textit{inhA} loci to determine the early detection of MDR-TB. Its sensitivity, specificity, PPV and NPV, turnaround time, and cost-effectiveness were calculated against the gold standard of sequencing. In the present study, phenotypically confirmed 94 MDR-resistant and 49 sensitive isolates were processed by HRM and sequencing to target \textit{rpoB}, \textit{katG}, and \textit{inhA} gene of \textit{M. tuberculosis}. Two and four isolates were genotypically found sensitive for RIF and INH, respectively, by both sequencing and HRM. Thus, \textit{rpoB} HRM assay was evaluated in 92 MDR isolates, whereas for INH, 90 isolates were the final sample size of evaluation of \textit{katG} and \textit{inhA} HRM assay in addition of 49 RIF and INH susceptible \textit{M. tuberculosis} isolates. HRM showed a high sensitivity and specificity of 98% each for mutation detection in \textit{rpoB} with one false-positive silent mutation L530 L detection as mutant and one false-negative Q490A due to mutation falling outside the HRM-targeted RRDR region of \textit{rpoB} as revealed by sequencing. Among 91 sequencing confirmed RIF-R isolates, HRM correctly identified 90 mutants by showing eight different melt curve cluster/patterns. All these mutations were nonsynonymous. On comparing our result with earlier published studies, we found HRM sensitivity of our study comparable to Choi \textit{et al.}, Ramirez \textit{et al.}, Ong \textit{et al.}, and Chen \textit{et al.}, showing sensitivity and specificity of 98.6%–100%, 89%–98%, and 94.4%–97.8%, respectively. Among 92 isolates, \textit{rpoB} exhibited maximum frequency of point mutation at codon position 531 (63 isolates, 68.47%) with predominance of TCG → TTG mutation in 61 isolates and infrequent mutation of TCG → TGG in two isolates. This was also found to be most common mutation in earlier studies. Other frequent mutations were noted in codon position 526 (12 isolates, 13.04%), 516 (10 isolates, 10.86%), and 513 (4 isolates, 4.34%), HRM also detected one rare mutations of CTG (Leu) → CGG (Arg) at codon 533. One mutant isolate by HRM was found with silent mutation at codon position 530 (CTG, Leu → CTC, Leu).

Although clinically this point mutation is nonsignificant, its overall clinical impact needs to be studied in greater detail with appropriate epidemiological studies as also supported in earlier studies with various codon noted for silent mutation included 511, 517, 521, 539, and 541. This is one of the limitations of HRM which needs to be addressed. Various earlier studies have tried to address this aspect by including genomic DNA samples with specific mutations and plasmid control to determine the presence of single mutations in clinical samples by HRM with 100% sensitivity. Two DST-proven resistant cases found susceptible by sequencing and HRM could be due to the reason of mutation falling outside targeted region of HRM and sequencing.

On the other hand, combined \textit{katG} and \textit{inhA} HRM showed the sensitivity of 90% (95% CI: 81.86–95.32) in detecting INH resistance as nine isolates with mutation in \textit{katG} gene were not detected by HRM. Sequencing analysis of these nine isolates revealed rare G299S mutation in five isolates and N341G mutation in one isolate of \textit{katG}, both were falling outside the target region of HRM. However, also inside the target region of \textit{katG} gene, HRM failed to detect dual mutation (AGC, Ser → GCC, Gly) at codon position 310 in three isolates. The most appropriate reason of this failure could be due to low Tm (melting temperature) difference causing no change in melt curve profile. Four phenotypic INH-R isolates showing sensitive result by HRM and sequencing could be due to either having mutation in other regions/codon of the targeted genes or various other genes (oxyR-ahp C, mab A, fur A, kas A, ini A/B/C, Rv1592c, etc.) which infrequently harbors mutations responsible for drug resistance as evident in earlier studies or drug efflux pumps mechanism. Simultaneous SNPs in all the three studied gene were seen in only three isolates, the effect of which needs to study further in terms of effect on level of resistance and pathogenicity.

The \textit{katG} and \textit{inhA} HRM was also found comparable to earlier studies. Previous studies of Ong \textit{et al.}, Choi \textit{et al.}, Ramirez \textit{et al.}, Chen \textit{et al.}, and Galarza \textit{et al.} reported sensitivity and specificity of 98%–83%, 84.1%–100%, 85%–98%, 95.7%–97.8%, and 98.7%–100%, respectively. Searching Indian literature, Yadav \textit{et al.} showed sensitivity and specificity of 93.1% and 100% for RIF resistance, while for INH resistance, these parameters were found 80% and 90%, respectively. Sensitivity of detection of MDR TB was reported as 92%.[28] Malhotra \textit{et al.} found sensitivity and specificity of 90.3% and 97.4% in detection of RIF resistance.[29] Showing high concordance with DST and sequencing results and having good sensitivity, specificity, less turnaround time, and low cost, HRM may be the simple molecular test for rapid detection of mutations encoding drug resistance. Other appealing features observed in HRM included minimal chances of cross-contamination, closed test, lesser DNA requirement (0.2 ng), and no requirement of hybridization. This study has an advantage over those published HRM studies which relied solely on the detection of RIF resistance to give
presumptive/surrogate indication of MDR as also evident from our finding of two mono RIF-resistant isolates that RIF resistance does not always lead to INH resistance. This view is also supported in earlier studies.\textsuperscript{[5,17]} Further INH susceptibility/ resistance pattern would be helpful to a great extent in deciding the treatment regimen for the patients. The identified MDR isolates may further be processed for fast-track second-line DST results to provide more effective treatment. HRM has an advantage of detecting other drug resistance also, namely, streptomycin, fluoroquinolones, and second-line injectable (capreomycin, amikacin, and kanamycin).\textsuperscript{[29]} These characteristics strongly support that HRM test could be of good clinical value, especially an effective low-cost alternative in a low-resource TB endemic country for effective management of MDR TB by detecting drug resistance mutation without the need of probes-based chemistry. With high NPV, it can be used for quick screening of suspected cases of MDR-TB due to low cost and rapid result in comparison to the current practice of waiting several weeks to obtain MDR status using conventional or automated DST. Eva green dye is used at a saturating concentration to allow the detection of mutational changes with different plot curves without handling post-PCR amplicon, thus reducing cross-contamination risk. Due to the low requirement of DNA, HRM can also be used directly in clinical samples, which although not evaluated in the present study, but with earlier published studies showing promising result, may be adequately considered.\textsuperscript{[30]}

**Conclusion**

In low-resource and high-endemic TB burden countries, HRM may be used in rapid screening of MDR cases of TB for effective management of the deadly MDR-TB by early initiation of evidenced-based antitubercular treatment and circumventing their high transmission rate.

**Significance of the study**

Since early and accurate diagnosis is the first step of effective management of TB and circumventing the transmission of MDR-TB, HRM may prove to be a vital molecular tool in screening of SNP-associated drug resistance.

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**Conflicts of interest**

There are no conflicts of interest.

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