

Genotypic Distribution and a Potential Diagnostic Assay of Multidrug-Resistant Tuberculosis in Northern Thailand

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Introduction: Knowledge of the prevalence and distribution of multidrug-resistant tuberculosis (MDR-TB) genotypes in northern Thailand is still limited. An accurate, rapid, and cost-effective diagnostic of MDR-TB is crucial to improve treatment and control of increased MDR-TB.

Materials and Methods: The molecular diagnostic assays named “RIF-RD” and “INH-RD” were designed to detect rifampicin (RIF) and isoniazid (INH) resistance based on real-time PCR and high-resolution melting curve analysis. Applying the ΔT_m cutoff values, the RIF-RD and INH-RD were evaluated against the standard drug susceptibility testing (DST) using 107 and 103 clinical *Mycobacterium tuberculosis* (Mtb) isolates from northern Thailand. DNA sequence analysis of partial *rpoB*, *katG*, and *inhA* promoter of 73 Mtb isolates, which included 30 MDR-TB, was performed to elucidate the mutations involved with RIF and INH resistance.

Results: When compared with the phenotypic DST, RIF-RD targeting *rpoB* showed sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 83.9, 98.6, 96.9, and 92.0%, respectively. The multiplex reaction of the INH-RD targeted both *katG* and *inhA* promoter showed high sensitivity, specificity, PPV, and NPV of 97.1, 94.2, 89.2, and 98.5%, respectively. Six patterns of *rpoB* mutation, predominately at codons 531 (50%) and 526 (40%) along with a rare S522L (3.33%) and D516V (3.33%), were detected. A single pattern of *katG* mutation (S315T) (63.3%) and four patterns of *inhA* promoter mutation, predominately -15 (C>T), were found. Approximately, 17% of MDR-TB strains possessed double mutations within the *katG* and *inhA* promoter.

Conclusion: Up to 86.7% and 96.7% of MDR-TB could be accurately detected by RIF-RD and INH-RD, emphasizing its usefulness as a low unit price assay for rapid screening of MDR-TB, with confirmation of INH resistance in low and middle-income countries. The MDR-TB genotypes provided will be beneficial for TB control and the development of drug-resistant TB diagnostic technology in the future.

Keywords: multidrug-resistant tuberculosis, *Mycobacterium tuberculosis*, high-resolution melting curve analysis, diagnosis, genotype

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Introduction

Tuberculosis (TB) remains a leading cause of morbidity and mortality worldwide. In 2018, an estimated 10 million people developed TB and up to 1.5 million deaths were globally reported.¹ The 30 high TB burden countries accounted for 87% of new TB cases and the greatest death occurred in low and middle-income countries (LMIC).¹ Thailand was one of 14 countries listed in the world health organization (WHO) “high burden country” for TB, multidrug-resistant (MDR)-TB and TB-HIV

co-infection.² Only 59 and 59.4% of treatment coverage for TB were reported in 2015 and 2016.^{1,2} Despite an increase in the treatment coverage in 2018 (~80%),¹ the effort to reduce under-diagnosis and under-reporting TB cases in Thailand is needed. TB burden in northern Thailand has been considered higher and more complex compared to other regions since it has been associated with MDR-TB, TB-HIV co-infection, and TB in migrant workers and border countries.³ However, data on MDR-TB genotypes and their distribution in this area have rarely been described.⁴ MDR-TB caused by *Mycobacterium tuberculosis* (Mtb) that resistant to rifampicin (RIF) and isoniazid (INH) is difficult to diagnose by culture method. Its treatment relies on the second-line drugs, which are difficult to procure and much more toxic and expensive than the first-line drugs. Failure to recognize and treat TB and MDR-TB has led to increased spread, mortality, and resistance to additional drugs, and extensively drug-resistant (XDR)-TB.^{5,6} To tackle this national and global crisis, the use of the molecular diagnostic tests including Xpert MTB/RIF has been recommended by WHO for the rapid detection of MDR-TB.¹

The majority of RIF resistance among MDR-TB in Asian countries is associated with the single-nucleotide substitution within the rifampicin resistance determining region (RRDR) of the β -subunit of bacterial RNA polymerase (*rpoB*) gene.⁷ Mutations of the catalase-peroxidase (*katG*) gene and the *InhA* (*inhA*) promoter region have been the most commonly reported determinants related to INH-resistance in Mtb worldwide.⁸ Several genotypic-based technologies for MDR-TB diagnosis such as the GeneXpert, the Genotype MTBDR plus assay, and the oligonucleotide probes and the line probe assay are currently available. However, their implementation in resource-limited countries is still limited due to costly reagents, complex procedures, and indistinctive results. Although the multi-probe real-time PCR provides detection of various mutations involved in RIF and INH resistance, they need several complicatedly-designed and costly probes.^{7,9-11} The rapid and low-cost loop-mediated isothermal amplification for the detection of *M. tuberculosis* complex (TB-LAMP) was endorsed by WHO but it cannot detect drug resistance.¹² Real-time PCR and high resolution melting (HRM) analysis has been applied for the detection of drug-resistant Mtb strains.¹³⁻¹⁷ Based on the variation of melting temperature (T_m) between mutant and wild type DNA sequences, a single-base mutation can be determined. The technique is greatly attractive since it is highly accurate, easy-to-perform, cost-effective, and capable of detecting unknown mutations within

2–3 hours.¹³⁻¹⁷ However, various real-time PCR platforms, reagents, platform-dependent analyses, and interpretations have hampered inter-laboratory result comparison and its application in routine diagnosis. Thus, two diagnostic tests for the rapid detection of MDR-TB were established and evaluated using clinical Mtb isolates from northern Thailand, which were analyzed in conjunction with MDR-TB genotypes.

Materials and Methods

Ethics and Biosafety

The ethical and biosafety issues of this study were approved by the Institutional Ethics Committee and Biosafety Committee, Chiang Mai University (approval no.: AMSEC 052/2559, CMU IBC A-003/2559).

Clinical *M. Tuberculosis* Isolates

During October 2015 to December 2017, the Ziehl-Neelsen acid-fast bacilli (AFB) staining and standard mycobacterial culture of collected sputa from suspected pulmonary TB patients were performed at a TB regional laboratory, Office of Disease Prevention and Control 1 (ODPC 1) Chiang Mai, Department of Disease Control, the Ministry of Public Health, Thailand, which covers 15 hospitals in 8 provinces in northern Thailand (Chiang Mai, Chiang Rai, Phayao, Nan, Phrae, Lampang, Lamphun, Mae Hong Son). One-hundred and seven clinical *M. tuberculosis* (Mtb) isolates were recovered on 2% Ogawa medium at 37°C for 4–8 weeks. The standard drug susceptibility testing (DST) of Mtb isolates against the first-line anti-TB agents was performed by the proportion method. Isolates were identified as resistant if >1% colony growth occurred on Middlebrook 7H10 agar comprising the critical drug concentrations (0.2 μ g/mL for INH, 1.0 μ g/mL for RIF, 2.0 μ g/mL for streptomycin, and 5.0 μ g/mL for ethambutol). Quality control was routinely performed during DST using the reference strain *M. tuberculosis* H37Rv (ATCC 27294).

Assay Design and Conditions

The nucleotide sequences of *rpoB*, *katG*, and *inhA* promoter (*fabG1-inhA*) in *M. tuberculosis* H37Rv (GenBank accession no. JX303332.1, X68081.1, and NC_000962.3) were used for primers design. Table 1 shows all primers used for RIF-RD and INH-RD assays established to detect RIF and INH resistance. The 20- μ L reaction mixture of either RIF-RD or INH-RD contained 1 \times LightCycler[®] 480 HRM master mix including ResoLight high-resolution melting dye (Roche

Table 1 The Oligonucleotide Primers Used in the RIF-RD and INH-RD Assays

Primer	Sequence (5'→3')	Target (size)	Reference
The RIF-RD assay			
rpoBF2	TCAAGGAGTTCTTCGGCAC	<i>rpoB</i> (123 bp)	This study
rpoB_R	CACGCTCACGTGACAGACC		Ong et al ¹⁴
The INH-RD assay			
katG-60FI	GGCTGGAAGAGCTCGTATGG	<i>katG</i> (124 bp)	This study
katG-60RI	CGTAGCCGTACAGGATCTCG		This study
inhA-FP	GGAAATCGCAGCCACGTTAC	<i>inhA</i> promoter (137 bp)	Peng et al ³⁰
inhA RI	GGTAACCAGGACTGAACGGG		Park et al ³¹

Diagnosics, Germany), 2.5 mM MgCl₂, 0.3 μM of *rpoB* primers for RIF-RD or 0.48 μM *katG-60* and 0.3 μM of *fabG1-inhA* primers for INH-RD, and 100 ng of DNA template. The genomic DNA of all *Mycobacterium* spp. were extracted using the DNA extraction reagents of Anyplex™ MTB/NTM Real-time Detection (Seegene, the Republic of Korea), according to the manufacturer's instruction. Genomic DNA of *M. tuberculosis* H37Rv (wide type) and two clinical isolates, Mtb A8 (RIF- and INH-susceptible) and Mtb MD62 (RIF- and INH-resistant), were included in each run of PCR and HRM analysis as references. Using the LightCycler® 96 real-time PCR system (Roche Diagnostics, Switzerland), the cycling profile was set as followings: 95°C for 3 min, and 40 cycles of 95°C for 10 s, 58°C for 15 s, and 72°C for 30 s. The amplicons were heated to 95°C for 1 min and cooled down to 40°C for 1 min proceeding HRM analysis with the temperature setting from 65 to 97°C, rising at 0.04°C/s with 25 acquisitions per degree Celsius.

Limit of Detection (LOD)

Genomic DNA of *M. tuberculosis* H37Rv was 10-fold serially diluted from 10 ng to 1 fg. To determine the assay LOD, the lowest amount of DNA detected by the assays was converted into an equivalent number of *Mycobacterium* bacilli based on *M. tuberculosis* H37Rv genome size.¹⁸

DNA Sequencing and Analysis

Amplification of the *rpoB*, *katG*, and *inhA* promoter region using primers covering 543 bp, 455 bp, and 455 bp of each gene was carried out by PCR.¹⁵ The DNA sequencing was performed using the ABI 3730XL DNA analyzer (Bioneer

co., Ltd., South Korea). DNA sequence and mutation were then analyzed using Bio Edit version 7.2.¹⁹

Statistical Analysis

The melting temperature (T_m) of each sample was acquired from real-time PCR and HRM analysis, then the T_m difference (ΔT_m) was calculated in comparison to *M. tuberculosis* H37Rv. The ΔT_m of all samples were analyzed by the Survival Analysis in XLSTAT (Addinsoft, New York, USA) to create the receiver operating characteristic (ROC) curve for determining the ΔT_m cutoff value.²⁰ The performance of the RIF-RD and INH-RD was evaluated using clinical Mtb isolates in northern Thailand. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated against data obtained from phenotypic DST and DNA sequencing.

Results

The Establishment of the RIF-RD and INH-RD

Based on real-time PCR and HRM analysis, the RIF-RD and INH-RD assays were established for the detection of MDR-TB. The reactions of both tests were optimized and can be run simultaneously using the annealing temperature of 58°C. Under the optimal conditions, RIF-RD successfully differentiated 37 phenotypically RIF-resistant (RIF-R) from 70 phenotypically RIF-susceptible (RIF-S) isolates (Figure 1A and B). The multiplexed INH-RD detected mutation of *katG* and *inhA* promoter and efficiently distinguished 34 phenotypically INH-resistant (INH-R) from 69 phenotypically INH-susceptible (INH-S) isolates (Figure 1C and D). The LOD of RIF-RD and INH-RD were equal at 1 pg of DNA per reaction, which is equivalent to approximately 210 genome copies of *M. tuberculosis* H37Rv.

The ROC Curve Analysis and the Assay Evaluation

RIF-RD and INH-RD were evaluated versus DST using 107 clinical Mtb isolates (70 RIF-S, 37 RIF-R) and 103 clinical Mtb isolates (69 INH-S, 34 INH-R). Using RIF-RD, the average ΔT_m of RIF-S and RIF-R isolates were at 0.08±0.08 and 0.53±0.22°C, respectively. The ROC curve analysis revealed the ΔT_m cutoff at 0.34°C (0.258–0.422, 95% CI) for distinguishing the RIF-R from RIF-S isolates (Figure 2A). The sensitivity, specificity, PPV, and NPV of RIF-RD against DST were then achieved at 83.9, 98.6,

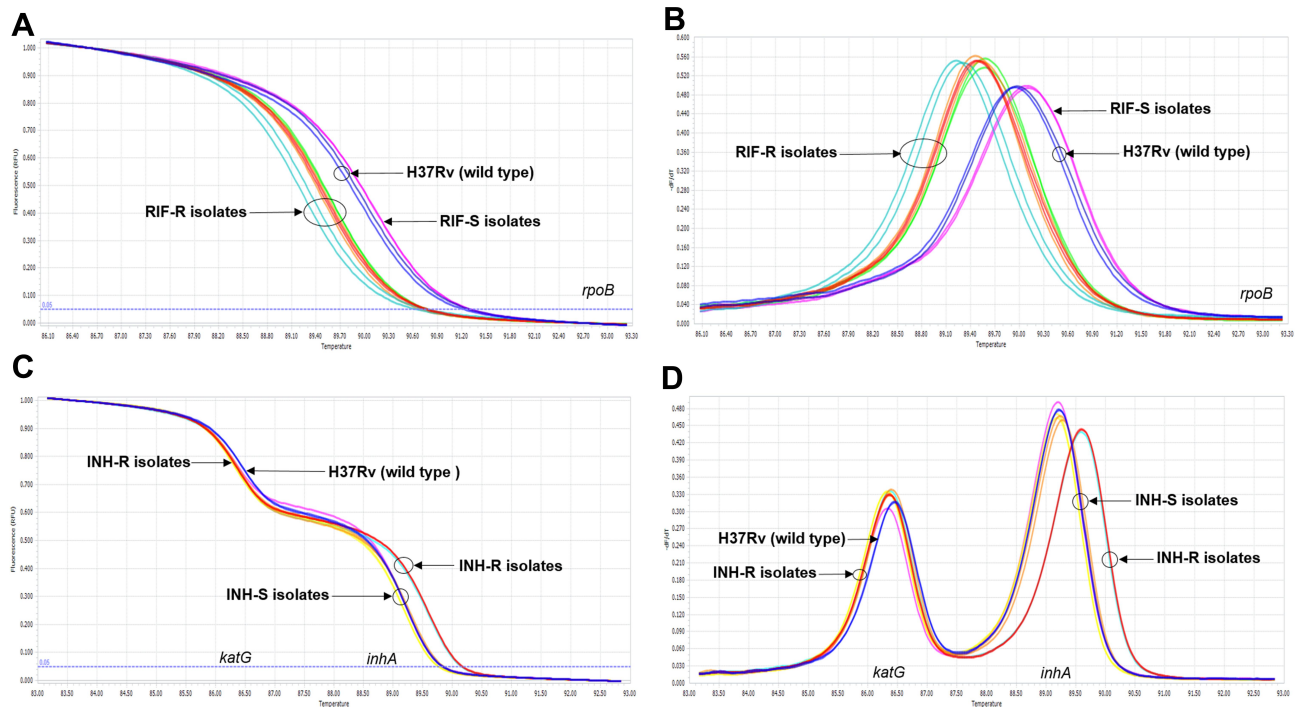


Figure 1 The differentiation between phenotypically drug-resistant and -susceptible *M. tuberculosis* clinical strains by the optimized RIF-RD (**A** and **B**) and INH-RD (**C** and **D**) assays using *M. tuberculosis* H37Rv as the reference (blue line): the normalized melting curve (**A** and **C**) and the normalized melting peak (**B** and **D**).

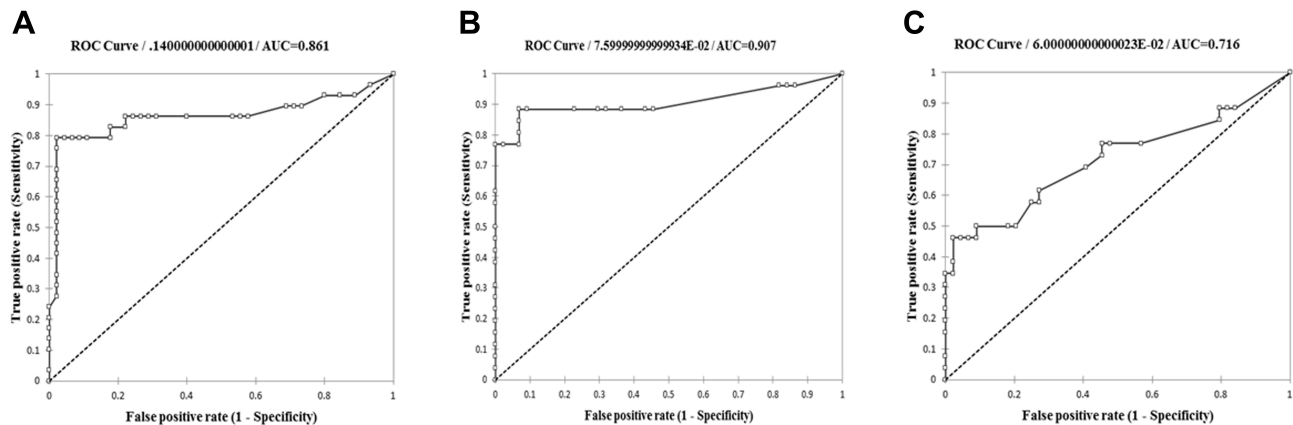


Figure 2 The ROC curve of *rpoB* (**A**), *katG* (**B**), and *inhA* promoter (**C**) applied for determining the ΔT_m cutoff to detect RIF and INH resistance in *M. tuberculosis* isolates.

96.9, and 92.0%, respectively. Using INH-RD, the average ΔT_m of INH-S and INH-R isolates were at 0.03 ± 0.02 and $0.12 \pm 0.06^\circ\text{C}$ for *katG*, and 0.04 ± 0.03 and $0.44 \pm 0.22^\circ\text{C}$ for *inhA* promoter amplicons. The ΔT_m cutoff for detection of INH resistance caused by *katG* and *inhA* promoter mutation were 0.06°C (0.034–0.086, 95% CI) and 0.10°C (0.05–0.15, 95% CI), respectively (Figure 2B and C). The sensitivity, specificity, PPV, and NPV of the INH-RD against DST were achieved at 97.1, 94.2, 89.2, and 98.5%, respectively. The evaluation against DNA sequencing showed the sensitivity, specificity, PPV, and NPV of

86.8, 100, 100, and 90.4% for RIF-RD ($n = 73$), and 96.7, 89.0, 78.4, and 98.5% for INH-RD ($n = 70$).

Distribution of MDR-TB Genotypes

Using the DNA sequencing method, six substitution mutation profiles of *rpoB* were discovered among 30 MDR-TB isolates in Thailand. The most predominant mutation, S531L, was found at 50% followed by H526Y (26.7%) and H526D (10%), respectively (Table 2). Interestingly, the S522L *rpoB* mutation rarely reported in MDR-TB was discovered in one isolate, and mutation was not detected in

one RIF-R isolate. Only S315T *katG* mutation was found at 63.3% but 4 mutation profiles were discovered in the upstream of *inhA* (-8, -9, -15, and -17). The most common *inhA* mutation at -15 (C>T) was detected at 16.67% (Table 2). About 66.7% of observed genotypes were the single mutation of *katG* or *inhA* promoter and about 16.7% were double mutations in both genes. Neither *katG* nor *inhA* mutation was detected in 5 isolates (16.7%). Albeit diverse mutation profiles observed, the RIF-RD and INH-RD efficiently differentiated different MDR-TB genotypes (Figure 3).

Discussion

Data interpretation of the RIF-RD and INH-RD based on the ΔT_m cutoff values was firstly established in this study. Test performance in terms of sensitivity, specificity, NPV, and PPV was evaluated by a ROC curve. The results showed that both RIF-RD and INH-RD could detect RIF resistance and INH resistance with high accuracy. The area under the curve (AUC) obtained indicated highly accurate detection of *rpoB* and *katG* mutations (AUC = 0.9) (Figure 2A and B), and moderately accurate detection of *inhA* promoter mutations (AUC = 0.7) (Figure 2C).²¹ Albeit the highly accurate

interpretation, there were incongruent results obtained. Among 37 RIF-R isolates, six were negative by the RIF-RD. Of these, four isolates contain the Class-III SNP (H526D), one isolate possesses the Class-IV SNP (D516V), which slightly affected the T_m . No mutation within the 543-bp sequenced *rpoB* in one RIF-R isolate suggesting alternative resistance mechanisms such as the efflux pump²² or decrease in drug uptake into cells.²³ DNA sequencing also confirmed a Class-I SNP nonsense mutation at codon 535 (C>T) in a fault positive sample. For INH-RD, only one phenotypically INH-R isolate was misidentified as susceptible but no mutation within the 455-bp targets was detected (data not shown). Its INH resistance probably was associated with the mutation of other genes such as *ahpC*, *emb*, *kasA*, and *ndh*.⁸ The INH-RD misidentified four INH-S isolates as resistant but DNA sequencing confirmed no mutation in both *katG* and *inhA* promoter. The increased ΔT_m may be affected by mixed amplicons derived from multiplexing in INH-RD.

The *rpoB* double mutations were not found among 30 MDR-TB isolates in northern Thailand in this study. A higher proportion of *rpoB* substitution mutation observed at codons 531 (50%) and 526 (26.7%) was comparable with previous reports in Thailand, Asian, and worldwide.^{7,11,24,25} Among 142 MDR-TB isolates in Thailand, 58% and 25.2% of *rpoB* mutation at codons 531 and 526 were reported.²⁴ However, the study of 34 MDR and XDR-TB isolates in 2005–2012 in northern Thailand reported eight *rpoB* mutations with no predominant codon including 588 (26.5%), 589 (26.5%), 526 (23.5%), 571 (20.6%), 531 (17.7%), 545 (17.7%), 564 (11.8%), and 574 (11.8%).⁴ Here, the S522L rarely found in *rpoB* mutant strains was discovered in an MDR-TB isolate. The S522L was found to involve in RIF resistance and rifabutin susceptibility. Additionally, the D516G-S522L double mutation was shown to be susceptible to RIF.²⁶

In this study, the rate of *katG* mutation at codon 315 among MDR-TB in northern Thailand (63.3%) is consistent with a global frequency (64%), and as low as 0.1–0.5% of INH-R strains mutated at other loci.⁸ A previous study in Thailand detected 84.5% of 110 MDR-TB isolates harboring *katG* mutation at codon 315, predominately S315T, and found only 2 isolates containing the mutation of *oxyR-ahpC* intergenic region.²⁷ However, a retrospective investigation during 2008–2011 found only 35.3% of 34 MDR and XDR-TB isolates from northern Thailand carrying the *katG* mutation at codon 315, together with others 10 loci.⁴ Four mutation patterns

Table 2 The Distribution of the *rpoB*, *katG*, and *inhA* Genotypes Among 30 MDR-TB Isolates in Northern Thailand

Mutation Profiles		No. of MDR-TB Isolates	Percentage (n = 30)
The single mutation			
<i>rpoB</i>	S531L (C>T)	15	50.00
	H526Y (C>T)	8	26.68
	H526D (G>C)	3	10.00
	D516V (A>T)	1	3.33
	S522L (C>T)	1	3.33
	H526R (A>G)	1	3.33
	ND	1	3.33
The single mutation			
<i>katG</i>	S315T (G>C)	14	46.67
<i>inhA</i>	-15 (C>T)	4	13.34
	-9 (T>C)	1	3.33
	-17 (G>T)	1	3.33
The double mutation			
<i>katG/ inhA</i>	S315T (G>C)/-8 (T>G)	3	10.00
	S315T (G>C)/-15 (C>T)	1	3.33
	S315T (G>C)/-17 (G>T)	1	3.33
	ND	5	16.67

Note: ND, mutation was not detected within the sequenced nucleotide length.

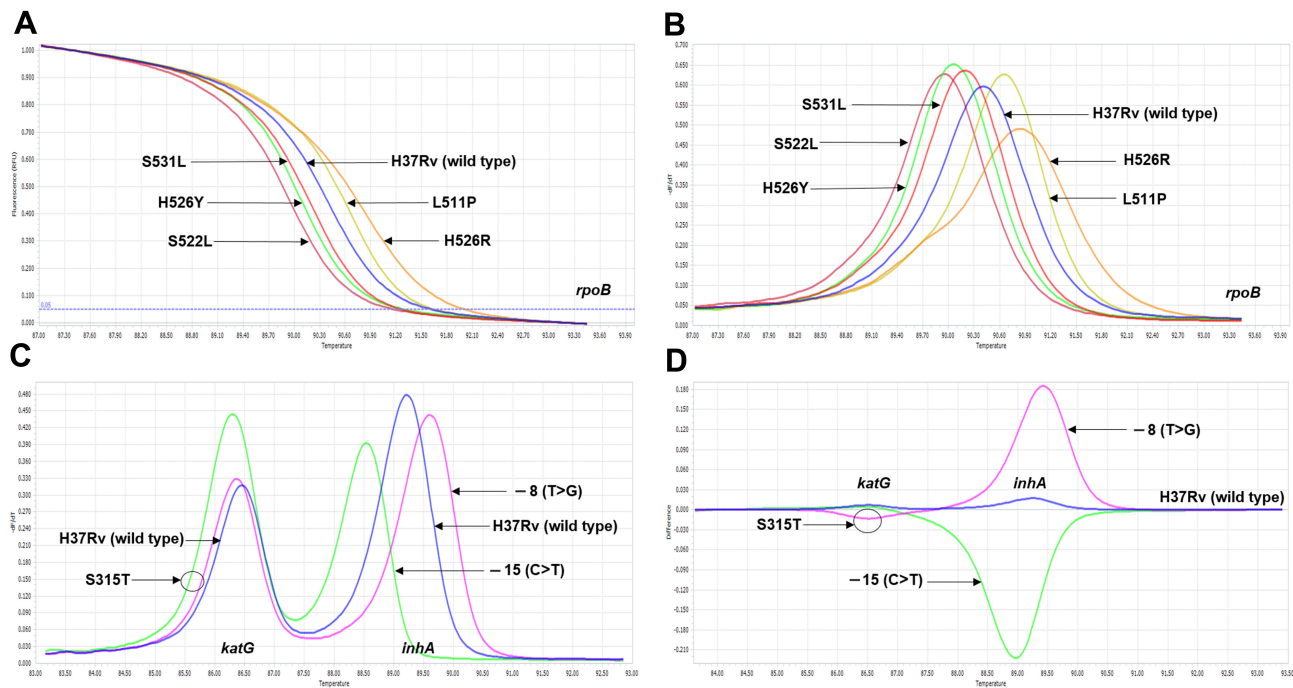


Figure 3 The differentiation among various mutation profiles observed in *rpoB*, *katG* and *inhA* promoter by the optimized RIF-RD (A and B) and INH-RD (C and D) assays using *M. tuberculosis* H37Rv as the reference (blue line): the normalized melting curve (A and C) and the normalized melting peak (B and D).

within the *fabG1-inhA* promoter were confirmed in this study. The rate of 20.6% for the most common genotypes, -15 (C>T), was comparable to the global frequencies: -15 (C>T) accounted for 19.2% of *inhA* promoter mutation.⁸ Similarly, mutations of the *inhA* promoter region and coding region were found at 15% (22/160) of INH-R isolates in Thailand, while -15 (C>T) was the predominant substitution mutation.²⁷

A real-time PCR combined with HRM analysis previously developed for RIF and INH resistance detection was evaluated against DNA sequencing using 217 *Mtb* isolates during 2007 and 2009 in South Korea.²⁸ The sensitivity and specificity for RIF resistance detection were 98.6 and 100%, while those for INH resistance were 84.1 and 100%.²⁸ Eight *rpoB* mutation patterns were detected including S531L (57.5%), S531E (15.1%), H526Y (8.2%), D516Y (8.2%), H526D (5.5%), H526L (2.7%), H526Q (1.4%) and D516V (1.4%). Two *katG* mutation profiles, S315T (67%) and D310A (1%) were found but only one mutation pattern in *inhA* promoter region, -15 (C>T) (33%), was reported.²⁸ Another test for RIF and INH resistance was evaluated against DNA sequencing using 167 Peruvian *Mtb* isolates.²⁹ High sensitivity and specificity of the test were 98.7 and 97.75% for RIF resistance detection and 98.7 and 100% for INH resistance detection. Up to 11 *rpoB* mutation patterns were found.²⁹ The most prevalent

rpoB mutation were S531L (67.9%), D516V (17.9%), and H526D (2.6%) but only one pattern of the S315T *katG* mutation and the -15 (C>T) *inhA* promoter mutation were discovered.²⁹ Although the same finding of *katG* mutation was observed, four mutation patterns of *inhA* promoter among isolates from Thailand were found in this study. Most studies calculated the sensitivity and specificity against DNA sequencing of *Mtb* isolates with known-mutation or susceptibility profiles. Here, we demonstrated the assay evaluation against both DNA sequencing and standard DST of blinded DNA samples. The genetic variation that responsible for RIF and INH resistance among *Mtb* and MDR-*Mtb* strains could be varied greatly depending on geographic locations, time of the investigation, and coverage of resistance gene target. The study of genotypic traits is therefore essential for better detection and elucidation of drug-resistant *Mtb* strains in a particular area at a specific point of time.

Up to 86.7% (26/30) and 96.7% (29/30) of all MDR TB isolates were detected by the RIF-RD and the INH-RD. DNA sequencing confirmed that 96.7% (29/30) of all MDR-*Mtb* studied possessed a single substitution within the targeted RRDR, which is consistent with the previous report.⁸ The *rpoB* mutation assay within this region thus may be useful as a screening test of MDR-TB in northern Thailand. The INH-RD detected *katG* and *inhA* promoter

mutation at 64% and 36%, respectively. This is correlated to the rates of *katG* and *inhA* promoter mutation among INH-R isolates reported earlier at 42–95% and 6–43% respectively while various mycobacterial genes associated with INH resistance have also reported.⁸ Further study on the genotypic distribution of more drug-resistant isolates might lead to the development of the genotypic-specific DST as well as baseline data on the epidemiology of MDR-TB in Thailand.

The established assays still require a costly real-time PCR system, processing in the laboratory, and well-trained staff. However, they provide detection of more diverse mutations among *rpoB*, *katG*, and *inhA* genes with a standardized interpretation of results using the ΔT_m cut-off, and a cheaper price per test (< \$7 for materials cost) compared to the probe-based real-time PCR, LPA, and GeneXpert MTB/RIF. The comparison of drug-resistance detection between the two assays and probe-based real-time PCR also demonstrated 100% concordance (data not shown). Besides, rapid analysis (2.5 hours from DNA extraction to results) and high throughput screening using 96-well PCR plates are possible. The nationwide application of the assays in a regional reference laboratory might be useful to improve treatment, surveillance, and control of MDR-TB in Thailand and Thailand border countries.

Conclusion

The genetic background among MDR-TB in northern Thailand provided here is essential for further TB control and the development of drug-resistant TB diagnostic technology. The establishment of the low cost, rapid and easy to perform screening/confirmation assays, both RIF-RD and INH-RD shall improve treatment, surveillance, and control of MDR-TB in LMIC, especially Thailand and Thailand border countries. The high-performance assay like the INH-RD is potentially useful for confirmation of MDR genotype as an additional/alternative test of commercially-available rifampicin-resistance assays.

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Disclosure

The authors report no conflicts of interest in this work.

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