Genotypic distribution of multidrug-resistant and extensively drug-resistant tuberculosis in northern Thailand

Background: Multidrug/extensively drug-resistant tuberculosis (M/XDR-TB) is a major public health problem, and early detection is important for preventing its spread. This study aimed to demonstrate the distribution of genetic site mutation associated with drug resistance in M/XDR-TB in the northern Thai population.

Methods: Thirty-four clinical MTB isolates from M/XDR-TB patients in the upper northern region of Thailand, who had been identified for drug susceptibility using the indirect agar proportion method from 2005 to 2012, were examined for genetic site mutations of katG, inhA, and ahpC for isoniazid (INH) drug resistance and rpoB for rifampicin (RIF) drug resistance. The variables included the baseline characteristics of the resistant gene, genetic site mutations, and drug susceptibility test results.

Results: All 34 isolates resisted both INH and RIF. Thirty-two isolates (94.1%) showed a mutation of at least 1 codon for katG, inhA, and ahpC genes. Twenty-eight isolates (82.4%) had a mutation of at least 1 codon for rpoB gene. The katG, inhA, ahpC, and rpoB mutations were detected in 20 (58.7%), 27 (79.4%), 13 (38.2%), and 28 (82.3%) of 34 isolates. The 3 most common mutation codons were katG 315 (11/34, 35.3%), inhA 14 (11/34, 33.3%), and inhA 114 (11/34, 32.4%). For this population, the best genetic mutation test panels for INH resistance included 8 codons (katG 310, katG 340, katG 343, inhA 14, inhA 84, inhA 86, inhA 114, and ahpC 75), and for RIF resistance included 6 codons (rpoB 445, rpoB 450, rpoB 464, rpoB 490, rpoB 507, and rpoB 508) with a sensitivity of 94.1% and 82.4%, respectively.

Conclusion: The genetic mutation sites for drug resistance in M/XDR-TB are quite variable. The distribution of these mutations in a certain population must be studied before developing the specific mutation test panels for each area. The results of this study can be applied for further molecular M/XDR-TB diagnosis in the upper northern region of Thailand.

Keywords: tuberculosis, drug resistance, MDR-TB, XDR-TB, genotype, mutation

Introduction

Tuberculosis (TB) is an important global problem, especially multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). In 2015, the world-wide estimation of MDR-TB cases and deaths was 80,000 and 250,000, respectively. MDR-TB is caused by Mycobacterium tuberculosis (MTB), which resists at least 2 drugs (isoniazid [INH] and rifampicin [RIF]). Pre-XDR-TB is a disease caused by the M. tuberculosis strain, which resists INH and RIF and either a fluoroquinolone or a second-line injectable drug, but not both. XDR-TB is defined as MDR-TB, which adds more resistance to any fluoroquinolone and at least 1 of 3 injectable second-line drugs (amikacin, capreomycin, and kanamycin [KAN]). Approximately 9.5% of MDR-TB...
patients developed XDR-TB. Unfortunately, about 20% of MDR-TB cases were undetectable. Thailand is among the countries with a TB burden, as it was ranked within the top 20 countries with TB in 2015.

Early and accurate detection of M/XDR-TB is important for effectively treating and preventing its transmission. However, the phenotypic drug susceptibility test (DST) takes weeks or months for culturing MTB and identifying the resisted drugs and requires a high level of microbiological safety. Diagnosis and treatment delays are among the major causes of the spread of the disease, which interferes with the TB control programs. A rapid molecular TB-DST test greatly increases the efficacy of MDR-TB treatment as the minimal turnaround time is <1 day.

As the distribution of mutation in drug-resistant genes is region specific, knowing the epidemiology of the genetic drug resistance of M/XDR-TB in certain areas would be beneficial for developing specific and rapid molecular tests, as well as treatment protocols, because the prevalence of such resistance differs in various populations. Many studies have focused on genetic drug resistance in MTB. INH is the main drug used; it is an effective anti-TB drug and has been used till date. The mechanism of INH resistance in MTB is associated with the mutations of katG, inhA, and ahpC. The katG mutation was found in high levels of INH resistance, and the inhA mutation was found at low levels or monoresistance to INH. RIF is combined with INH as the main anti-TB drug, and it is inferred with transcription by the DNA-dependent RNA polymerase by binding to the β-subunit hindering transcription organisms related to rpoB 531 mutation, which destroys the organisms.

In this study, we examined the distribution of drug-resistant gene mutations, including katG, inhA, ahpC, and rpoB, in M/XDR-TB isolates in the northern Thai population. Then, we identified the best mutation test panels for INH and RIF resistance.

**Materials and methods**

Two hundred and sixty-one M/XDR-TB isolates first diagnosed with MDR-TB or XDR-TB from patients between January 2005 and June 2012 were retrieved from an archive of the Laboratory of the Office of Disease Prevention and Control Region 10 (DPC10). According to the World Health Organization (WHO) criteria, MDR isolates resisted at least INH and RIF; and XDR isolates resisted INH, RIF, ofloxacin (OFX), and KAN. DPC10 hosts a regional TB laboratory, covering 8 provinces in the north of Thailand, and the isolates were subcultured. Only 34 specimens (9.5%) were grown in 5 mL of 7H9 broth supplemented with PANTA in 6 weeks and 3% Ogawa within 8 weeks. The bacterial agents were further examined for phenotypic DST and DNA sequencing.

**Phenotypic drug susceptibility test**

Thirty-four growing M/XDR-TB isolates were tested for first- and second-line drug resistance (INH, RIF, OFX, and KAN) using the proportion method with Lowensentzen Jensen (LJ) medium at DPC10 laboratory. The DOTS-plus DST was performed according to the WHO guidelines, and all suspected TB and MDR-TB patients were investigated using 3 methods: 3 direct acid-fast bacilli sputum smear examinations, a *Mycobacterium* culture, and first-line DST during the first diagnosis. Additionally, at the same time, a molecular test was carried out in order to confirm *M. tuberculosis*. DST was determined using the indirect agar proportion method with INH, RIF, streptomycin, and ethambutol. If there was resistance to INH and/or RIF, a second-line anti-TB DST was performed with OFX and KAN. For our study, LJ medium was supplemented individually with anti-TB drugs. INH (0.2 µg/mL), RIF (40.0 µg/mL), OFX (2.0 µg/mL), and KAN (30 µg/mL) were repeated in order to confirm that the results were the same as the previous results; if the results were not the same then they were excluded from the study.

**DNA extraction**

Thirty-four M/XDR-TB isolates were cultured on solid media (LJ and OGAWA). Chromosomal DNA was extracted using the MolecuTech REBA MTB-MDR 2011 commercial kit. Briefly, 1 loop (0.2 µL diameter sterile inoculating loop) of the cultured bacteria was resuspended in 50 µL of DNA extraction solution. The solution was vortexed for 1 min, boiled for 10 min, and centrifuged (13,000 rpm) for 3 min at room temperature. Then, 5–10 µL of the supernatant was transferred to a microtube (cryovial tube). The purified DNA pellet was stored at 4°C.

**Sequencing method**

The polymerase chain reaction (PCR) and DNA sequencing parts were carried out at Macrogen Laboratory, Seoul, Republic of Korea. Four genetic loci, *katG*, *inhA*, *ahpC* (INH), and *rpoB* (RIF), were amplified by PCR, as shown in Table S1. The used primers are shown in Table S1. The amplified products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA).
**Analysis**

**DNA sequencing analysis**

The sequencing data obtained from the ABI3730XL DNA analyzer were investigated for the presence or absence of mutations by alignments with the corresponding nucleotide sequences of *M. tuberculosis* (H37RV) using the NCBI nucleotide blast program.

**Data analysis**

The phenotypic DST and genetic site mutation for the DST data sets were compiled using an Excel 2010 database. Statistical analysis was performed using STATA version 11.0. The resistant genes and genetic site mutations were presented according to frequency and percentage.

**Results**

Among the 34 growing M/XDR-TB isolates, 24 (70.5%) MDR-TB, 9 (26.5%) Pre-XDR-TB, and 1 (3.0%) XDR-TB were identified. All of the isolates were resistant to both INH and RIF. The frequency and patterns of genetic loci mutation are demonstrated in Tables 1 and 2. Table 3 exhibits the raw data of genetic mutation in all 34 isolates. Mutations were found, including *katG* in 20 isolates (58.8%), *inhA* in 27 isolates (79.4%), *ahpC* in 13 isolates (38.2%), and *rpoB* in 28 isolates (82.3%). INH-resistant gene mutations were found in 32 isolates (94.1% sensitivity). RIF-resistant gene mutations were identified in 28 isolates (82.4% sensitivity).

One isolate revealed no mutation for the studied genetic loci (no. 18). Two isolates showed no mutation for *katG*, *inhA*, or *ahpC*. (no. 2 and 18). For the INH-resistant genes, a single-gene mutation was found in 10 isolates (29.4%), 2 for *katG*, 7 for *inhA*, and 1 for *ahpC*. Double mutation was found in 16 isolates (47.1%), 10 for *katG/inhA*, 4 for *inhA/ahpC*, and 2 for *katG/ahpC*. Triple *katG/inhA/ahpC* mutation was seen in 6 isolates (17.6%). Regarding the RIF-resistant

**Table 1** Frequency of genetic site mutations of *katG*, *inhA*, *ahpC*, and *rpoB* genes in 34 M/XDR-TB isolates

| SNP       | InhA n (%) | katG n (%) | ahpC n (%) | rpoB n (%) |
|-----------|------------|------------|------------|------------|
| katG229   | 1 (2.9)    | 11 (32.4)  | 4 (11.8)   | 8 (23.5)   |
| katG300   | 1 (2.9)    | 1 (2.9)    | 4 (11.8)   | 6 (17.7)   |
| katG302   | 1 (2.9)    | 8 (23.5)   | 4 (11.8)   | 6 (17.7)   |
| katG308   | 3 (8.8)    | 7 (20.6)   | 3 (8.8)    | 4 (11.8)   |
| katG310   | 3 (8.8)    | 9 (26.5)   | 7 (20.6)   | 7 (20.6)   |
| katG312   | 4 (11.8)   | 8 (23.5)   | 6 (17.7)   | 4 (11.8)   |
| katG314   | 1 (2.9)    | 3 (8.8)    | 7 (20.6)   | 9 (26.5)   |
| katG315   | 1 (2.9)    | 11 (32.4)  | 5 (14.7)   | 9 (26.5)   |
| katG320   | 1 (2.9)    | 14 (41.3)  | 11 (32.4)  | 9 (26.5)   |
| katG340   | 4 (11.8)   | 10 (29.4)  | 4 (11.8)   | 6 (17.7)   |
| katG343   | 7 (20.6)   | 1 (2.9)    | 1 (2.9)    | 1 (2.9)    |

**Abbreviation:** M/XDR-TB, multidrug/extensively drug-resistant tuberculosis.

**Table 2** Distribution of *Mycobacterium tuberculosis* genetic mutations with isoniazid and rifampicin resistance

| Gene mutation codon | n | %   |
|---------------------|---|-----|
| katG                |   |     |
| No mutation         | 14| 41.3|
| katG 315            | 9 | 26.7|
| katG 320            | 1 | 2.9 |
| katG 312            | 1 | 2.9 |
| katG 343            | 1 | 2.9 |
| katG 315 and katG 343| 1 | 2.9 |
| katG 308 and katG 310| 1 | 2.9 |
| katG 312 and katG 343| 2 | 5.9 |
| katG 308, katG 315, and katG 340| 1 | 2.9 |
| katG 315, katG 340, and katG 343| 1 | 2.9 |
| katG 299, katG 300, katG 302, katG 310, and katG 312| 1 | 2.9 |
| InhA                |   |     |
| No mutation         | 7 | 20.6|
| inhA 14             | 6 | 17.7|
| inhA 25             | 1 | 2.9 |
| inhA 86             | 1 | 2.9 |
| inhA 114            | 5 | 14.8|
| inhA 14 and inhA 114| 4 | 11.8|
| inhA 84 and inhA 114| 1 | 2.9 |
| inhA 78, inhA 81, and inhA 84| 1 | 2.9 |
| inhA 78, inhA 81, and inhA 86| 1 | 2.9 |
| inhA 78, inhA 84, and inhA 86| 1 | 2.9 |
| inhA 14, inhA 78, inhA 81, and inhA 84| 1 | 2.9 |
| inhA 78, inhA 81, inhA 84, and inhA 86| 1 | 2.9 |
| inhA 81, inhA 84, inhA 86, and inhA 94| 1 | 2.9 |
| inhA 78, inhA 81, inhA 84, inhA 86, and inhA 94| 2 | 5.9 |
| ahpC                |   |     |
| No mutation         | 21| 61.8|
| ahpC 10             | 1 | 2.9 |
| ahpC 75             | 1 | 2.9 |
| ahpC 10 and ahpC 12 | 1 | 2.9 |
| ahpC 12 and ahpC 20 | 1 | 2.9 |
| ahpC 25 and ahpC 76 | 6 | 17.7|
| ahpC 10, ahpC 20, and ahpC 22| 1 | 2.9 |
| ahpC 12, ahpC 20, and ahpC 22| 1 | 2.9 |
| ahpC 10, ahpC 12, ahpC 20, and ahpC 22| 1 | 2.9 |

(Continued)
Table 2 (Continued)

| Gene mutation codon | n  | %   |
|---------------------|----|-----|
| rpoB                |    |     |
| No mutation         | 6  | 17.7|
| rpoB 445           | 2  | 5.9 |
| rpoB 508           | 1  | 2.9 |
| rpoB 464           | 2  | 5.9 |
| rpoB 450           | 3  | 8.8 |
| rpoB 490           | 2  | 5.9 |
| rpoB 507           | 1  | 2.9 |
| rpoB 445 and rpoB 508 | 1 | 2.9 |
| rpoB 507 and rpoB 508 | 1 | 2.9 |
| rpoB 445 and rpoB 483 | 1 | 2.9 |
| rpoB 464 and rpoB 483 | 1 | 2.9 |
| rpoB 464, rpoB 464, and rpoB 504 | 1 | 2.9 |
| rpoB 445, rpoB 490, and rpoB 493 | 1 | 2.9 |
| rpoB 450, rpoB 490, and rpoB 493 | 1 | 2.9 |
| rpoB 445, rpoB 464, and rpoB 493 | 1 | 2.9 |
| rpoB 450, rpoB 490, rpoB 483, and rpoB 493 | 1 | 2.9 |
| rpoB 445, rpoB 464, rpoB 490, rpoB 483, and rpoB 493 | 1 | 2.9 |

gene, no rpoB mutation was found in 6 isolates (no. 5, 8, 11, 15, 8, and 34).

The common mutated genetic loci found in M/XDR-TB were katG 315 (n=12, 35.3%), inhaA 14 (n=11, 32.4%), inhaA 114 (n=11, 32.4%), inhaA 84 (n=9, 26.5%), rpoB 507 (n=9, 26.47%), and rpoB 508 (n=9, 26.47%).

Discussion

The frequency and distribution of drug-resistant mutations are variable across regions and countries (Table 4). The frequency of katG mutation ranged from 58.5% to 93.7%, and the most commonly mutated katG locus was katG 315. The reported frequency of inhaA mutation was 4.7%–79.4%, and the most commonly mutated inhaA locus was inhaA 15. The frequency of ahpC mutation was relatively low (9.1%–38.2%) and was extremely

Table 3 Raw distribution of genetic site mutations of katG, inhaA, ahpC, and rpoB genes in M/XDR-TB isolates

| No. | katG 299 | katG 300 | katG 302 | katG 308 | katG 310 | katG 312 | katG 314 | katG 315 | katG 320 | katG 340 | katG 343 | katG 34 | inhaA 14 | inhaA 78 | inhaA 81 | inhaA 84 |
|-----|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 1   | Y        | Y        | Y        | Y        | Y        |         |         |         |         |         |         |         |         |         |         |         |
| 2   |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 3   |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 4   |         |         |         |         |         | Y        | Y        |         |         |         |         |         |         |         |         |         |
| 5   |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 6   |         |         |         |         |         |         | Y        |         |         |         |         |         |         |         |         |         |
| 7   |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 8   |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 9   |         |         |         |         |         |         |         |         |         |         |         |         | Y        |         |         |         |
| 10  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 11  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 12  |         |         |         |         |         |         |         |         |         |         |         |         |         | Y        |         |         |
| 13  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 14  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 15  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 16  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 17  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 18  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 19  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 20  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 21  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 22  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 23  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 24  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 25  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 26  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 27  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 28  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 29  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 30  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 31  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 32  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 33  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| 34  |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |

Notes: -, no mutation; Y, presence of mutation; gray column, proposed mutation test panels.

Abbreviation: M/XDR-TB, multidrug/extensively drug-resistant tuberculosis.
variable. The frequency of rpoB mutation was relatively high (80.8%–97.0%), predominantly at the locus rpoB 531.

Genomic tests or multiple genetic loci tests are not suitable for resource-limited countries. Developing test panels for M/ XDR-TB drug-resistant genes would be more cost-effective if the test panels of the selected genetic loci provide high sensitivity and specificity. However, as the distributions of the gene mutations across the regions varied, a deeper analysis should be considered in further studies. The distribution of mutation patterns should be examined in each geographical areas before routine diagnostic services being implemented. According to the results of this study, we selected 8 INH-resistant gene mutation loci (katG 310, katG 340, katG 343, inhA 14, inhA 84, inhA 86, inhA 114, and ahpC 75) and 6 RIF-resistant gene mutation loci (rpoB 445, rpoB 450, rpoB 464, rpoB 490, rpoB 507, and rpoB 508) as the best panels for genetic drug resistance tests. Both the panels were able to detect all isolates with at least 1 mutation site for any katG/inhA/ahpC mutation and rpoB mutation. The sensitivities of the proposed INH resistance mutation test was 94.1% (32/34), while that of the RIF resistance mutation test was 82.4% (28/34).

**Limitations**

The limitations of this study were the small sample size and the retrospective design. Only 34 isolates were available from a total of 261 isolates. Another weak point was that there was no control group, such as non-M/XDR-TB isolates.

**Conclusion**

The results of this study confirm the presence of genetic drug-resistant mutations in the M/XDR-TB isolates in upper northern Thailand. We showed that our mutation patterns

### Table 3

| inhA 86 | inhA 94 | inhA 114 | ahpC 10 | ahpC 12 | ahpC 20 | ahpC 22 | ahpC 75 | ahpC 76 | rpoB 445 | rpoB 450 | rpoB 464 | rpoB 490 | rpoB 507 | rpoB 508 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
were different from other parts of Thailand and other countries. The distribution of these mutations in certain populations must be studied before developing specific mutation test panels for each area. These data and proposed mutation panels can be applied for further molecular M/XDR-TB diagnosis in the upper northern region of Thailand.

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Author contributions
All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure
All the authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Primers used for sequencing

| Gene  | Primer | Nucleotide sequencing (5′–3′) | Product size (pb) | Temperature (°C) | Reference |
|-------|--------|-------------------------------|-------------------|------------------|-----------|
| katG  | MtkatGf| ACCGAGGCTGCTCCGCTGG          | 168               | 94°C – 20 s       | Afanas’ev\(^1\) |
|       | MtkatGr| CAGCTCCCACCTCGTAGCCGT        |                   | 50°C – 20 s 70 cycles |           |
|       |        |                               |                   | 72°C – 20 s       |           |
| inhA  | MtfabGf| GCCTCGTGGCCCAGAAAGG          | 320               | 94°C – 20 s       | Afanas’ev\(^1\) |
|       | MtfabGr| CTCCGGATCCACGGTGAGGG         |                   | 56°C – 20 s 70 cycles |           |
|       |        |                               |                   | 72°C – 20 s       |           |
| ahpC  | MtfabGr| GCCTCGTGGTGTCGACTGGT         | 359               | 95°C – 40 s 15 min | Valvatne\(^2\) |
|       | ahpC1 F| CTCCGGATCCACGGTGAGGG         |                   | (start) 94°C – 40 s 30 cycles |           |
|       |        |                               |                   | 57°C – 40 s 1 min |           |
|       |        |                               |                   | 72°C – 40 s 15 min |           |
|       | ahpC2 R| CGCAACGTGACCTGGCTCATA        | 321               | 94°C – 20 s       | Afanas’ev\(^1\) |
|       | MrtpoBf| GAGGGCATCACCGCAGAC           |                   | 59°C – 20 s 70 cycles |           |
|       | MrtpoBr| GGTACGCGGTTCGAGTGAAC         |                   | 72°C – 20 s       |           |

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