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Novel mutations detected from drug resistant *Mycobacterium tuberculosis* isolated from North East of Thailand

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(A. Sangka).

Abstract

The emergence of drug-resistant tuberculosis is a major global public health threat. Thailand is one of the top 14 countries with high tuberculosis, tuberculosis co-infected with Human Immunodeficiency Virus (HIV) and multi-drug resistant tuberculosis rates. Immediate detection of drug-resistant tuberculosis is necessary to reduce mortality and morbidity by effectively providing treatment to ameliorate the formation of resistant strains. Limited data exist of mutation profiles in Northeastern Thailand. Here, 65 drug-resistant *Mycobacterium tuberculosis* isolates were used to detect mutations by polymerase chain reaction (PCR) and DNA sequencing. In the *katG* gene, mutations occurred in 47(79.7%) among 59 isoniazid resistant samples. For *rpoB* gene, 31 (96.9%) were observed as mutations in 32 rifampicin resistant isolates. Of 47 *katG* mutation samples, 45 (95.7%) had mutations in *katG*315 codon and 2 (4.3%) showed novel mutations at *katG*365 with amino acid substitution of CCG-CGG (Pro-Arg). Moreover, 18 (58.1%) mutations at *rpoB*531, 11 (35.5%) mutations at *rpoB*516, 10 (32.3%) mutations at *rpoB*526 and 1 (3.2%) mutation at *rpoB*533 were found between 31 rifampicin resistant samples. Common and novel mutations of the *rpoB* and *katG* genes in Northeastern Thailand were generated. DNA sequencing showed high accuracy, while conventional polymerase chain reaction could be applied as an initial marker for screening rifampicin and isoniazid drug-resistant *Mycobacterium tuberculosis* in Northeastern Thailand. Mutations reported here should be considered when developing new molecular diagnostic methods for implementation in Northeastern Thailand.

Keywords: drug-resistant, *Mycobacterium tuberculosis*, mutation, *rpoB*, *katG*

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) bacteria is a serious global infectious disease even though diagnosis and treatment are available. About one fourth of population (10 million people) in the world is suffering from this disease, with death rates of 1.4 million people in 2019 (World Health Organization 2020). The emergence of multi-drug resistant TB (MDR TB), described as resistant to at least rifampicin and isoniazid, as the two most commonly applied powerful first-line anti-TB drugs, is also a major public health threat worldwide (Lange et al. 2019) (Jang and Chung 2020). Globally in 2019, an estimated 465,000 incident cases were reported as MDR and rifampicin resistant (RR) TB, while 3.3% and 13% of new cases and 18% and 17.4% of previously treated cases were diagnosed with MDR/RR TB and isoniazid (INH) resistant TB, respectively (World Health Organization 2020). Thailand is one of the top 14 countries with high TB, TB/HIV and MDR-TB and had 150 TB cases per 100,000 population in 2019 (World Health Organization 2020) (Anukool et al. 2020). Immediate detection and accurate identification of MDR-TB is necessary to reduce mortality and morbidity by effectively providing treatment, to ameliorate the formation and spread of resistant strains (Jaksuwan et al. 2017)(Nimri et al. 2011)(Prammananan et al. 2008). The phenotypic DST method is the gold standard for detection of drug resistance but this takes 2-3 months to yield results and also increases the risk of bacterial contamination and cross infection by non-tuberculous mycobacterial infection. To shorten the turnaround time, rapid molecular diagnostic polymerase chain reaction (PCR) and DNA sequencing methods were selected in this study (Genestet et al. 2020; Li et al. 2020; Jang and Chung 2020). The distribution of drug-resistant genes varies according to geographical locations and identifying the mutation patterns of MDR-TB is important to optimize the treatment protocol (Jaksuwan et al. 2017) (Seifert et al. 2015). However, limited data exist of mutation profiles in Northeastern Thailand.

A better understanding of drug-resistant mechanisms and the most commonly found mutation codons of rifampicin (RIF) and isoniazid (INH) is required. RIF is one of the most effective anti-TB drugs. Its mode of action involves binding to the β -subunit of RNA polymerase, thereby inhibiting the elongation of messenger RNA (Palomino and Martin 2014). The mechanism of RIF resistance in MTB is associated with mutation of the *rpoB* gene (Isakova et al. 2018). Some 96% of the mutation codons of RIF resistant MTB isolates mostly occur in 81 bp spanning codons (507-533), called the rifampicin resistance determining region (RRDR). Among these codons, *rpoB* 516, 526 and 531 provided the highest level of resistance with percentage 70-95% (Palomino and Martin 2014)(Tajbakhsh et al. 2018). Another powerful drug for MTB is isoniazid and mutations in several genes such as *katG*, *inhA*, *oxyR*, *kasA*, *ahpC* and *ndh* are responsible for INH drug resistance. Most INH resistance is caused by the *katG* gene that codes for the catalase-peroxidase enzyme, a key factor in mycolic acid biosynthesis. The most common mutation in INH-resistant strains is *katG*315 (50-95%) (Gupta et al. 2013) (Pandey et al. 2017) (Ravibalan et al. 2015).

Here, we examined the mutations of drug-resistant MTB strains emphasizing on the *rpoB* and *katG* genes in Northeastern Thailand populations using PCR and DNA sequencing methods.

2. Materials and Methods

Sample sources and processing: A total of 357 MTB isolates were randomly selected from the TB laboratory at the Office of Disease Prevention and Control Center (DPC) 7th, 8th, 9th and 10th (Khon Kaen Province, Udon Thani Province, Nakhon Ratchasima Province and Ubon Ratchathani Province, respectively) between 2013 and 2016 in Northeastern Thailand. Among these samples, 92 isolates were found with drug resistance (rifampicin and isoniazid) by testing with the standard proportion method in all DPC centers (Rueangsak et al. 2020). In this study, 65 drug-resistant MTB isolates were used for detection of mutation patterns in Northeastern Thailand. All isolates were confirmed as MTB by observing culture positive on Lowenstein-Jensen medium and using immunochromatographic assay (ICA) kit (SD Bioline TB Ag MPT64 rapid test) to detect and amplify real-time PCR.

DNA extraction: Genomic DNA of the 65 drug-resistant isolates of *Mycobacterium tuberculosis* (26 resistant to both RIF and INH, 33 mono-resistant to isoniazid and 6 mono-resistant to rifampin) were extracted using the CTAB (cetyl trimethylammonium bromide) method (Van Embden et al. 1993). In two microcentrifuge tubes, each 400 μ L of bacterial cell suspension was transferred. After that, both tubes were put on heat block with the temperature at 80°C for 20 mins. After adding all chemical reagents, the DNA pellets were re-dissolved in 50 μ L of TE buffer and stored at -20 °C and -70 °C until required for further use. The estimation of DNA concentration was done by using a spectrophotometer (NanoDrop 2000c, USA) with the absorbance at a wavelength of 260 nm.

PCR amplification: Conventional polymerase chain reaction was used to determine mutations in 4 regions for the *rpoB* gene codons 516, 526, 531 and 533 and 1 region for the *katG* gene codon 315 using specific primers (Table 1) (Nurpermatasari et al. 2018) (Ahmed et al. 2013). For each condition, the preparation of master mix concentration and cycling temperature were modified from the methods of (Allegui et al. 2012) (Tajbakhsh et al. 2018) (Farnia et al. 2010).

Amplification reactions for the *rpoB*516 and *katG*315 were performed in a final volume of 50 μ L containing 0.5 μ L of 2.5mM dNTPs, 5 μ L of 10XBuffer, 0.25 μ L of 5U/ μ L Taq polymerase, 1.66 μ L of 50mM MgCl₂, 40.59 μ L sterile DI water, 0.5 μ L of each forward and reverse 10 μ M primers and 1 μ L of 20ng of genomic DNA. Similar concentrations of all reagents were also used for amplifying the *rpoB*531 and *rpoB*526 genes with a final volume of 25 μ L composed of 0.25 μ L of dNTPs, 2.5 μ L of Buffer, 0.2 μ L of Taq polymerase, 0.5 μ L (for 531) and 1 μ L (for 526) of MgCl₂, 18.55 μ L (for 531) and 18.05 μ L (for 526) of sterile DI water, 1 μ L of each primer and 1 μ L of DNA. For the *rpoB*533 amplification, the PCR reaction was performed in a final volume of 25 μ L containing the same concentrations of the above reagents accompanied by dNTPs (2 μ L), Buffer (2.5 μ L), Taq (0.25 μ L), MgCl₂ (2.75 μ L), DI water (13 μ L), forward primer (0.5 μ L), reverse primer (2 μ L) and DNA (2 μ L).

The DNA amplifications were performed in a thermal cycler, FlexCycler2, (Analytik Jena AG, Germany). Cycling temperatures for the *rpoB* 516 and *katG* 315 genes were initial denaturing at 96 °C for 3 min; 23 cycles of 95 °C for 50 s, 68 °C for 40 s, and 72 °C for 1 min; and final elongation at 72 °C for 7 min. For *rpoB* 531 and 526 genes, the following temperature cycles were used: initial denaturing at 95°C for 5 min, 40 cycles of 94 °C for 1 min, 58 °C (for 531) and 55 °C (for 526) for 1 min, 72 °C for 1

min, and a final extension at 72 °C for 10 min. Furthermore, initial denaturing at 95 °C for 5 mins; 40 cycles of 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s; and final elongation at 72 °C for 7 min were used as cycling temperatures for amplifying the *rpoB* 533 primer. All PCR fragments were analyzed by agarose gel electrophoresis using 1.5% agarose. The gels were exposed to UV light with photos taken by a GeneFlash gel documentation system (Syngene, USA).

DNA sequencing: Two primers: *rpoB*-F (5'-TACGGTCGGCGAGCTGATCC-3') and *rpoB*-R (5'-TACGGCGTTTCGATGAACC-3') were used to amplify a 411-bp fragment (1119 – 1532bp) of the *rpoB* gene, containing 81-bp (RRDR region). The region of the 435 bp (669-1103bp) *katG* gene was amplified with a set of primers F-GCAGATGGGGCTGATCTACG and R-AACGGGTCCGGGATGGTG. Before sequencing, PCR amplification reactions were modified following (Farnia et al. 2010) and (Jaiswal et al. 2017). Final volumes of 50 µL were used for the *rpoB* and *katG* genes. For the *rpoB* gene, sterile DI water 32.9 µL, 10Xbuffer 5 µL, 5U/µL Taq Polymerase 0.5 µL, 2.5mM dNTPs 4 µL, 50mM MgCl₂ 1 µL, 10 µM Forward and Reverse Primers 0.8 µL each and 4 µL of 20ng DNA were used, while sterile DI water (25.75 µL), 10Xbuffer (6.25 µL), 5U/ µL Taq Polymerase (0.5 µL), 2.5mM dNTPs (4 µL), 50mM MgCl₂ (2 µL), 10 µM forward and reverse primers (3.75µL for each) and 4 µL of 20ng DNA were used to amplify the *katG* gene.

PCR conditions were as follows: initial denaturation of 95 °C for 5 min (*rpoB*) and 95°C for 3 min (*katG*), followed by 40 cycles of 95 °C for 30 s, annealing at 69 °C for 30 s, extension of 72 °C for 30 s and final extension of 72 °C for 7 min for the *rpoB* gene followed by 35 cycles of 95 °C for 45 s, annealing at 64 °C for 45 s, extension of 72 °C for 1 min and final extension of 72 °C for 5 min for the *katG* gene. The PCR products were analyzed by electrophoresis on 2% agarose gel under ultraviolet light. After amplification, the PCR products containing specific genes and specific portions were sent to a sequencing company (Ward Medic), including the gel purification step.

Sequence Analysis: The sequencing data obtained from the company were aligned with the wild type sequences of *rpoB* and *katG* genes for H37Rv (GenBank accession NC_000962.3) using the BioEdit bioinformatic tool. Then, mutation codons and types of mutation in the samples were examined. For the *rpoB* gene, the nucleotide numbering system was based on *E. coli*.

Two isolates with novel mutation in the *katG*365 gene can be accessed (GenBank accession no. MZ093466, MZ093467).

3. Results

Out of 357 MTB isolates, 92 samples (25.8%) were found with drug resistance, as already described by (Rueangsak et al. 2020). Of the 92 drug-resistant samples, 38 (41.3%) were multi-drug resistant, while 46 (50%) and 8 (8.7%) were mono-resistant to isoniazid and mono-resistant to rifampicin, respectively. Sixty-five drug-resistant samples (26 multi-drug resistant, 33 mono-resistant to isoniazid and 6 mono-resistant to rifampicin) were used to find mutations in the hot spot regions of the *rpoB* and *katG* genes.

PCR amplification results

To detect mutations in the *rpoB*516,526,531,533 and *katG*315 genes, each reaction for amplifying target size was optimized using the H37RV reference strain as the DNA template (**Fig. 1** Optimization results of *rpoB*516, 526, 531, 533 and *katG*315 primers by PCR shown by 1.5% agarose gel electrophoresis). Fifty-nine mono-resistant isoniazid isolates were detected by the *katG*315 primer to determine the mutations. Among the 56 isoniazid resistant samples, 45 (80.4%) were identified as mutation in the *katG*315 gene. The remaining 3 samples were not differentiated by PCR.

Consequently, 32 mono-resistant rifampicin isolates were tested to observe mutations using all *rpoB* primers individually. Of 32 R to RIF samples, the *rpoB*516 gene mutation was found in 10 (31.2%), *rpoB*526 mutation was observed in 5 (15.6%) and 11 (34.4%) were identified as mutation in the *rpoB*531 gene. There were no mutation isolates with the *rpoB*533 primer. Therefore, the *rpoB*531 gene mutation had the highest percentage followed by *rpoB*516 and *rpoB*526.

Twenty-six MDR isolates were detected to observe combined mutations of both genes using *rpoB*516,526,531,533 and *katG*315 primers. A total of 13 out of 26 (50%) MDR cases showed mutations in both genes, while 4 isolates harbored no mutation in MDR samples. Mutation in the *katG* gene alone was found in 8 samples, whereas 1 sample was found to have mutation in the *rpoB* gene alone in MDR isolates. Six samples showed no mutation in mono R to INH and 3 samples of mono R to INH were not differentiated by PCR. Three samples were found as no mutation in mono R to RIF samples. Hence, 15 samples for the *rpoB* gene and 14 samples for the *katG* gene were selected for DNA sequencing to detect mutation sites not detected by PCR.

DNA sequencing results

PCR amplification for DNA sequencing of the *rpoB* and *katG* genes was optimized using the H37Rv reference strain as the DNA template (**Fig. 2** Optimization results of *rpoB* and *katG* genes by PCR shown by 2% agarose gel electrophoresis). Fifteen isolates that exhibited the *rpoB* gene with no mutation and 14 isolates that showed no mutation at *katG*315 codon by PCR were sequenced. Our analysis detected 5 mutated positions distributed among 4 codons within RRDR of the *rpoB* gene. Of the 15 isolates, 7 had mutation at codon 531 with amino acid changes at the position of TCG-TTG (Ser-Leu), while mutation at codon 526 of CAC-CGC (His-Arg) occurred in 3 isolates and changes of CAC-TAC (His-Tyr) occurred in 2 isolates. Moreover, 1 mutation at codon 516 GAC-GTC (Asp-Val) was identified, while a mutation at codon 533 displayed CTG-CCG (Leu-Pro) amino acid change. No mutation was detected in 1 isolate.

Among the 14 isolates that showed no mutation at *katG*315 codon, 2 isolates had mutation at codon 365 CCG-CGG (Pro-Arg) (**Fig. 3** Chromatogram of the nucleotide substitution (CCG-CGG) detected in two novel isolates at *katG*365 codon). These were recognized as novel mutations and no mutations were observed in 12 samples. The above 3 samples that were not differentiated by PCR revealed no mutation with the sequencing method.

Distribution of mutation profiles based on PCR and DNA sequencing methods

The *katG* gene mutations occurred in 47 (79.7%) of the 59 R to INH samples. For the *rpoB* gene, 31 (96.9%) were observed as mutations in 32 R to RIF isolates. Of the 47 *katG* mutation samples, 45 (95.7%) had mutations in *katG*315 codon and 2 (4.3%) showed mutations in *katG*365 codon (**Fig. 4 (a)** Mutation frequency percentage in the *katG* gene among 47 R to INH isolates). Moreover, 18 (58.1%) at *rpoB*531, 11 (35.5%) mutations at *rpoB*516, 10 (32.3%) mutations at *rpoB*526 and 1 (3.2%) mutation at *rpoB*533 were found between 31 R to RIF samples in this study (**Fig. 4 (b)** Mutation frequency percentage in the *rpoB* gene among 31 R to RIF isolates). Out of 26 MDR isolates, combined mutations of the *rpoB* and *katG* genes were found in 22 (84.6%) samples, while no mutation was observed in 1 (3.8%) and only *rpoB* mutation occurred in 3 (11.5%) samples (**Fig. 4 (c)** Mutation genes frequency percentage in 26 MDR isolates). Finally, geographical distribution of the *rpoB* and *katG* gene mutation samples found in each province of Northeastern Thailand were shown. (**Fig. 5** Geographical distribution of the *rpoB* and *katG* genes mutation samples found in each province of Northeastern Thailand region).

Discussion

This is the first study determining the distribution of *Mycobacterium tuberculosis* mutation genes in Northeastern Thailand. We found 41.3% as MDR, 50% as R to INH and 8.7% as R to RIF among 92 drug-resistant isolates collected from 4 DPC centers (17 provinces) in Northeastern Thailand that has 20 provinces. Among the 92 isolates, 65 drug-resistant samples (26 MDR, 33 R to INH and 6 R to RIF) were used to detect the mutation genes of *rpoB* and *katG* using PCR and DNA sequencing methods. RIF resistance is caused by mutation in the *rpoB* gene, encoding the β -subunit of the DNA-dependent RNA polymerase (Jureen et al. 2006) (Chaves et al. 2000). More than 95% of *rpoB* mutations

in RIF-resistant clinical isolates have been found within the rifampicin resistant determining region (RRDR) (González et al. 1999). Our findings revealed that 96.9% of *rpoB* gene mutations were observed within the RRDR region. Our result was similar to a report in Bangladesh that also showed 96.43% of *rpoB* gene point mutation in the RRDR (Mistri et al. 2016). Other studies have documented the presence of common and novel *rpoB* mutations outside the RRDR (Mani et al. 2001) (Aparna Lingala et al. 2010). According to our size of product, we only determined the most prevalent mutation codons between the positions 454-591 including the RRDR region. Inside the 81-bp RRDR, mutations within codons 516, 526 and 531 are responsible for up to 90% of RIF-resistant strains and recognized as the most frequent codons (Pang et al. 2013) (Williams et al. 1994). Out of the *rpoB* gene mutation isolates in our study, 58.1% were detected at *rpoB531*, 35.5% at *rpoB516* and 32.3% at *rpoB526*. Therefore, the most prevalent mutation codon was *rpoB531*, consistent with other findings in the Kyrgyz Republic (64.8%) (Isakova et al. 2018), Shanghai (53.8%) (Fan et al. 2003) and North India (66.7%) (Makadia et al. 2012). Most studies in China (Yue et al. 2003), Vietnam (Caws et al. 2006) and Singapore (Lee et al. 2005) exhibited the *rpoB526* codon as the second highest mutation frequency, different from our results. Moreover, only 1 (3.2%) sample was found to have mutation in *rpoB533* (Leu-Pro). This codon 533 was also detected in a study in Ratchaburi Province, Thailand as a novel mutation; however, different amino acids indicated *rpoB533* (Leu-Arg) (Jintaridh et al. 2014). In accordance with our study, reports from Thailand by (Prammananan et al. 2008) and Taiwan by (Qian et al. 2002) displayed *rpoB533* (Leu-Pro) with percentages of 2.1 and 3, respectively. Furthermore, double point mutation was determined in 7 samples and triple point mutation was identified in 1 sample in our study. In our research, *rpoB531* codon that expressed highest mutation percentages, could be applied as an indicator to investigate rifampicin resistant *Mycobacterium tuberculosis* isolates in this area.

Nearly all rifampicin-resistant strains are further resistant to other drugs, especially to isoniazid. Thus, rifampicin resistance is recognized as a surrogate marker for MDR-TB (Traore et al. 2000). Detection of resistance for another drug in this study was isoniazid that inhibits the synthesis of mycolic acids and acts as a prodrug that is activated by the catalase-peroxidase enzyme, *katG* (Jena et al. 2014) (Timmins and Deretic 2006). Our report showed that *katG* gene mutation was found at the rate of 79.7%, in which 95.7% had mutations in *katG315* codon and 4.3% harbored mutation in *katG365* codon. The percentage of 79.7% for the *katG* gene mutation was consistent with earlier studies in Thailand (Boonaiam et al. 2010) (Suthum et al. 2020) and other Southeast Asian Countries (Ismail et al. 2016) (Caws et al. 2006) (Valvatne et al. 2009) (Liu et al. 2018) (Cheng et al. 2021). The prevalence of *katG315* mutation varied according to the geographical region: Southeast Asia (78.4%) (Seifert et al. 2015), Vietnam (85.3%) (Hang et al. 2019), China (59.4%) (Wu et al. 2006), the Netherlands (53%) (Van Soolingen et al. 2000), Japan (22%) (Ando et al. 2010) and Singapore (26%) (Lee et al. 1999). Furthermore, our results revealed higher percentage of *katG315* mutation in R to INH isolates than in MDR strains (53.3% and 46.7%, respectively). Two isolates of novel mutation at *katG365* (GenBank accession no. MZ093466, MZ093467) with the amino acid substitution of CCG-CGG (Pro-Arg) were exhibited in our report. As previously mentioned, the *katG315* gene mutation provided higher percentages, and this mutation codon could be utilized as an indicator for the detection of isoniazid resistance to *Mycobacterium tuberculosis* isolates in this region.

After PCR amplification, 15 samples of R to RIF that did not show any mutation with primers of the PCR method were selected for *rpoB* gene sequencing to discover more mutation codons. These 15 isolates showed single point mutation within RRDR of the *rpoB* gene in 14 and no mutation in 1 sample. In 15 isolates of R to RIF, we found 7 samples at *rpoB531* mutation with the amino acid substitution of TCG-TTG (Ser-Leu). Moreover, mutation at codon 526 of CAC-CGC (His-Arg) occurred in 3 isolates and changes of CAC-TAC (His-Tyr) occurred in 2 isolates only, while the amino acid change pattern of mutation at codon 516 was GAC-GTC (Asp-Val). Furthermore, mutation at codon 533 displayed CTG-CCG (Leu-Pro) amino acid change. *katG* sequencing was performed in the 14 isolates that showed the *katG* gene, with no mutation in 5 MDR samples, 6 R to INH samples and 3 samples not detected by PCR. Among the 14 isolates, 12 exhibited no mutation and 2 displayed mutation in 365 codons (Pro-Arg). The 3 samples that were not detected by PCR revealed no mutation with the sequencing method. Mutation patterns performed by DNA sequencing in this study showed missense mutation in all isolates.

According to Fig. 5, mutations of the *rpoB* and *katG* genes in each province were widely distributed. The *katG315* gene mutation samples were fully distributed throughout Northeastern Thailand and detected in every province except Bueng Kan that also showed no mutation samples. Moreover, *rpoB531* gene mutation samples were also widespread and found in nearly every province except for Bueng Kan, Chaiyaphum, Nakhon Ratchasima, Yasothon and Maha Sarakham. Higher distribution of mutation codons was found in provinces located near the Laos border. In Sakon Nakhon and Amnat Charoen Provinces, 2 isolates (one in each province) showing novel mutation were detected, while 1 isolate of *rpoB533* gene mutation was found in Mukdahan Province.

When comparing PCR and DNA sequencing methods, discordant results were found for detection of the *rpoB* gene but not for the *katG* gene. PCR did not identify 7 isolates of *rpoB531* mutation, 5 samples of *rpoB526* mutation, 1 sample of *rpoB516* mutation and 1 sample of *rpoB533* mutation, possibly because the isolates contained heterogeneous populations of bacteria with mutated and wild type *rpoB* alleles, leading to amplification of the wild type PCR product (Ullah et al. 2019). No mutations were detected in 1 isolate of R to RIF and 12 isolates of R to INH by both PCR and DNA sequencing. Mutations outside the *rpoB* and *katG* genes were not considered in this study. The DNA sequencing method showed greater sensitivity and specificity than PCR but the latter is more appropriate for use as a screening method to obtain faster results in low resource countries. Our results showed similar percentages of mutation codons reported in Thailand and Southeast Asian Countries except for *katG315* percentage and having novel mutation of *katG365* codon.

Study limitations were as follows. Gene mutation of *katG*, *ahpC*, *inhA* and *kasA* can cause isoniazid resistance (Zhang et al. 1992); however, we only detected *katG* gene mutation that provided high-level resistance. Even though we identified the *katG* gene mutation, the amino acid change pattern of this gene mutation could not be performed by sequencing, especially in *katG315*. Furthermore, a larger sample size would further validate our findings. We were unable to identify the entire genes of *rpoB* and *katG* because we focused on the most prevalent mutation sites for sequencing. Therefore, further studies should sequence the entire *rpoB* and *katG* genes to obtain more accurate results. The mutations reported here should be considered in the development of new molecular diagnostic methods for implementation in Northeastern Thailand.

4. Conclusions

Even though DNA sequencing has great accuracy, conventional PCR methods can be applied as an initial marker for the screening of rifampicin and isoniazid in Northeastern Thailand. Our results showed that *rpoB531* codon and *katG315* codon expressed highest mutation percentages and could be utilized as indicators to investigate rifampicin and isoniazid resistant *Mycobacterium tuberculosis* isolates in this area. Two isolates with novel mutation at *katG365*, with amino acid substitution CCG-CCG (Pro-Arg) were exhibited in our report. The mutations reported here should be considered in the development of new molecular diagnostic methods for implementation in Northeastern Thailand. These novel techniques could reduce mortality and morbidity and provide effective treatment to ameliorate the formation of further resistant strains and spread of disease in Northeastern Thailand.

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Declarations

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Figures

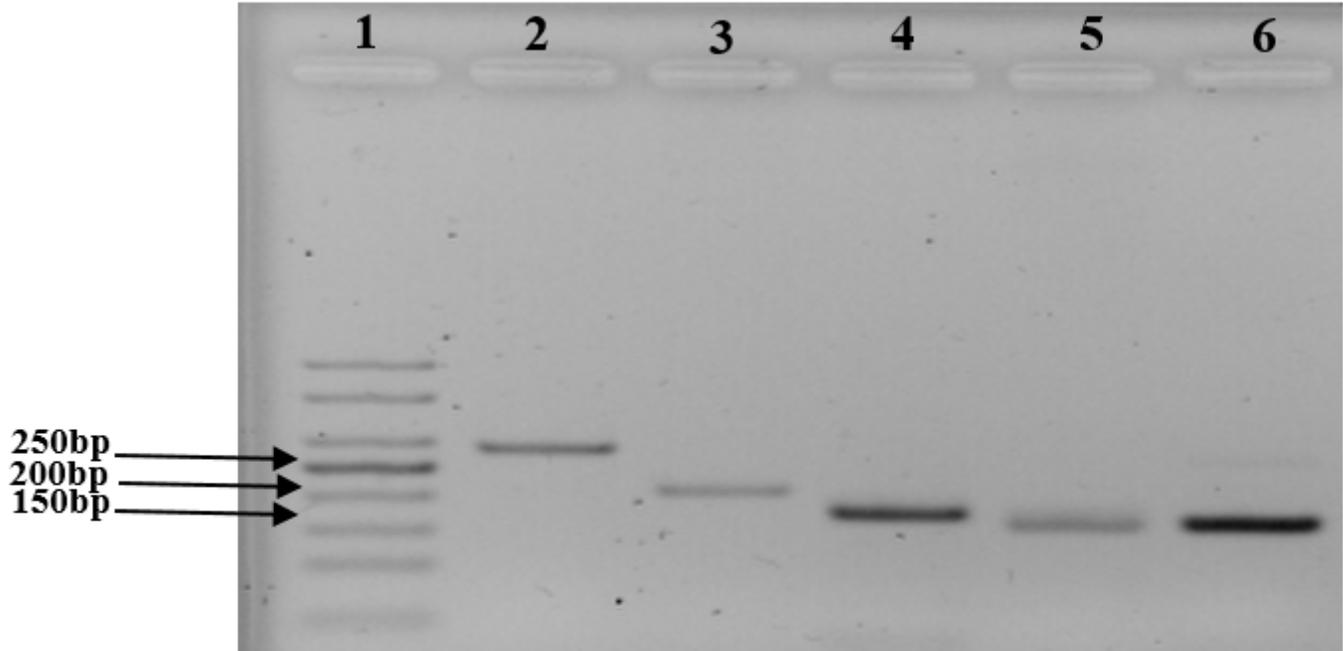


Fig. 1

Figure 1

Optimization results of rpoB516, 526, 531, 533 and katG315 primers by PCR shown by 1.5% agarose gel electrophoresis

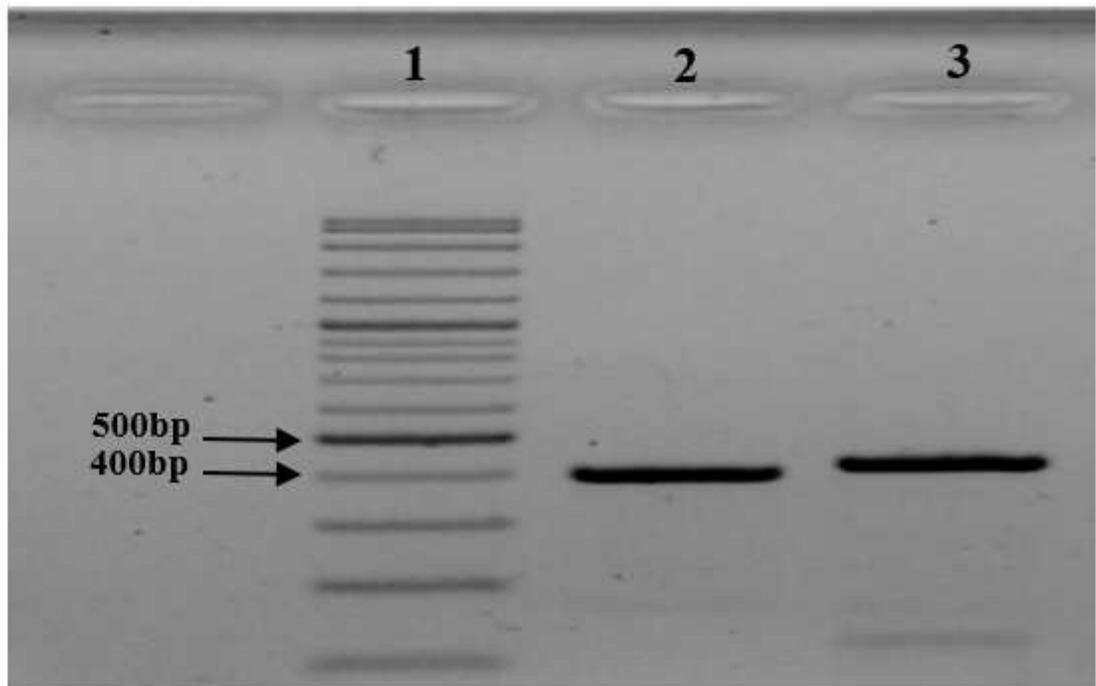


Fig. 2

Figure 2

Optimization results of *rpoB* and *katG* genes by PCR shown by 2% agarose gel electrophoresis

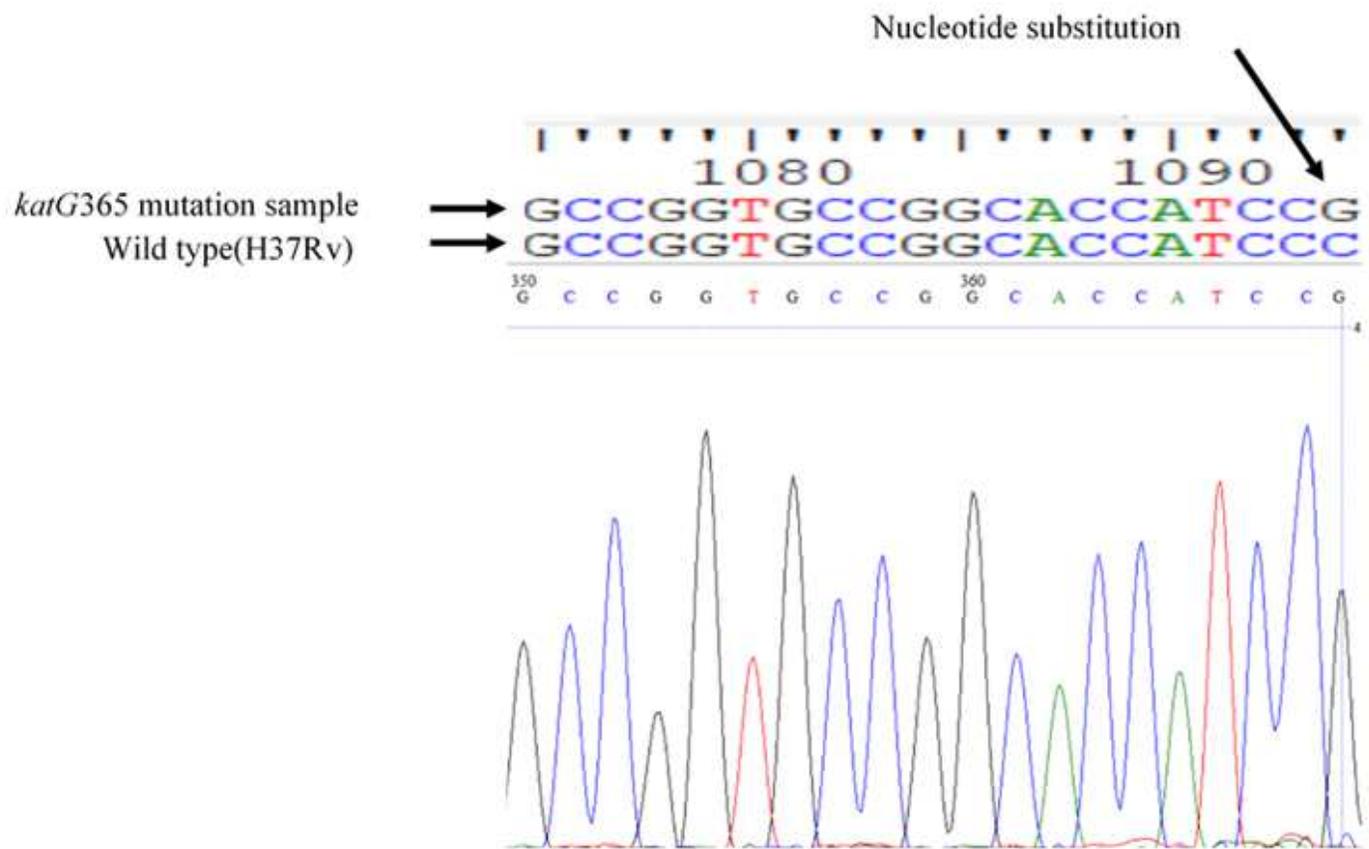


Fig. 3

Figure 3

Chromatogram of the nucleotide substitution (CCG-CGG) detected in two novel isolates at *katG*365 codon

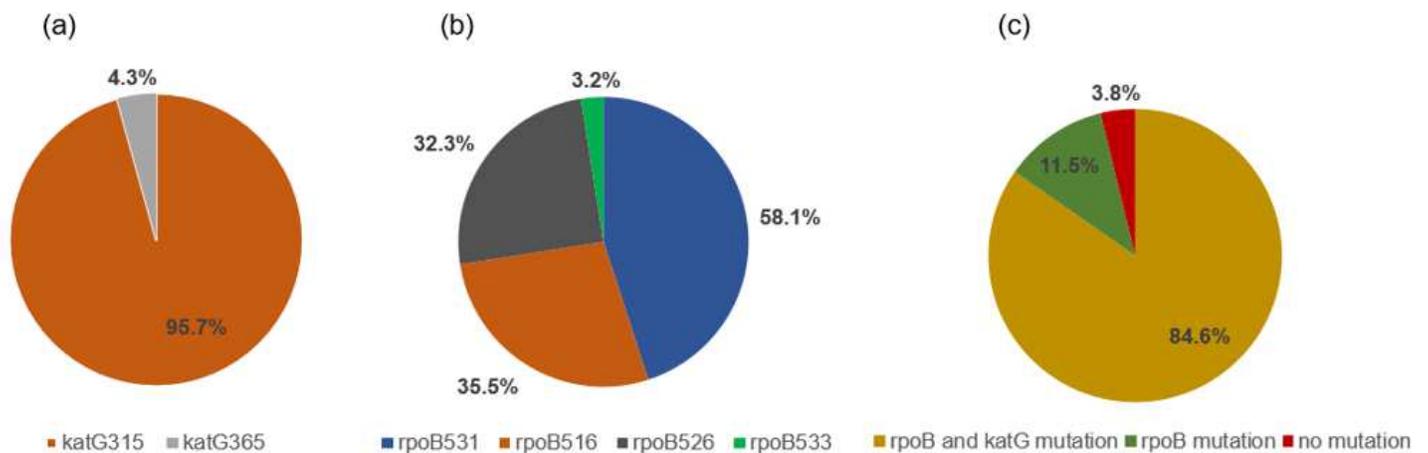


Figure 4

(a) Mutation frequency percentage in the katG gene among 47 R to INH isolates). Moreover, 18 (58.1%) at rpoB531, 11 (35.5%) mutations at rpoB516, 10 (32.3%) mutations at rpoB526 and 1 (3.2%) mutation at rpoB533 were found between 31 R to RIF samples in this study (Fig. 4 (b) Mutation frequency percentage in the rpoB gene among 31 R to RIF isolates). Out of 26 MDR isolates, combined mutations of the rpoB and katG genes were found in 22 (84.6%) samples, while no mutation was observed in 1 (3.8%) and only rpoB mutation occurred in 3 (11.5%) samples (Fig. 4 (c) Mutation genes frequency percentage in 26 MDR isolates

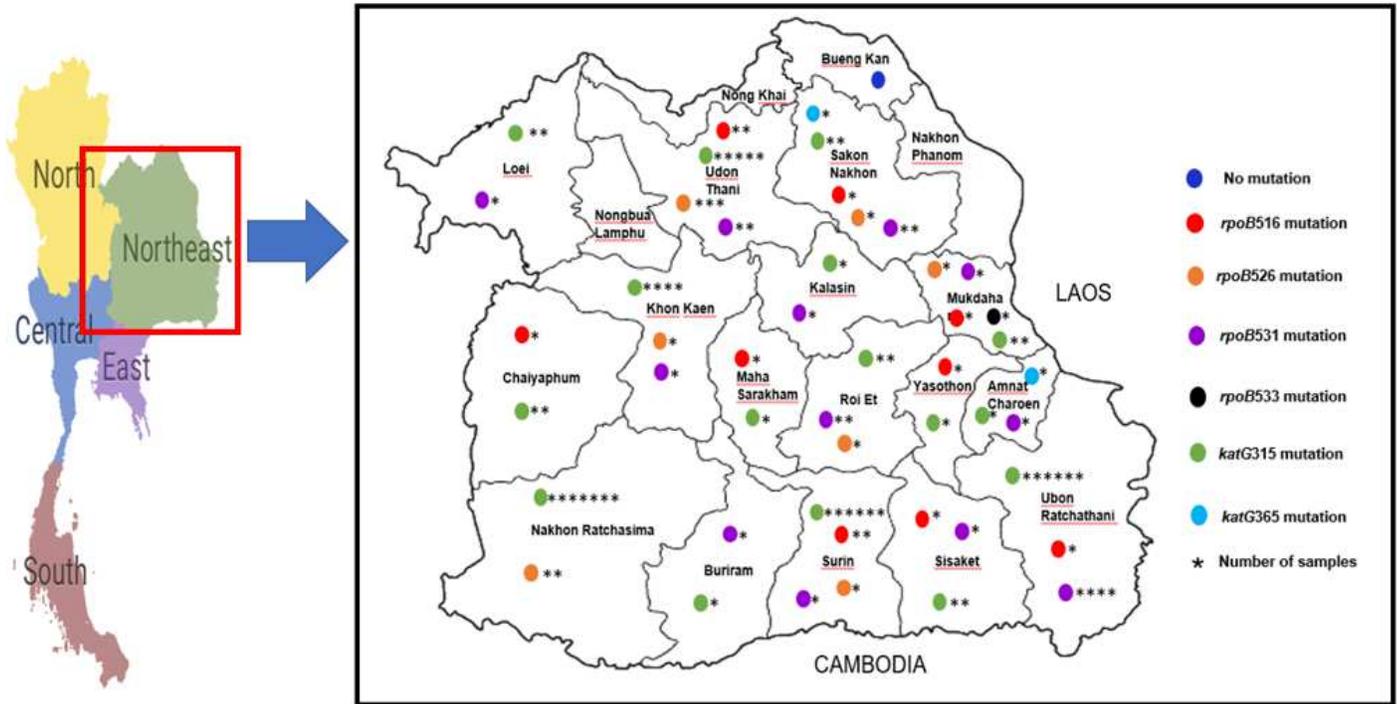


Fig. 5

Figure 5

Geographical distribution of the rpoB and katG genes mutation samples found in each province of Northeastern Thailand region