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Original Article

Characterization of *katG, inhA, rpoB* and *pncA* in *Mycobacterium tuberculosis* isolates from MDR-TB risk patients in Thailand

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Abstract

Introduction: Multidrug-resistant tuberculosis (MDR-TB) is commonly found in Thailand especially in the public health region 5, the Western region of Thailand. This study's aim was to characterize *katG, inhA, rpoB* and *pncA* genes in *Mycobacterium tuberculosis.*

Methodology: One hundred strains of *Mycobacterium tuberculosis* (MTB) were isolated from sputum samples of MDR-TB risk patients in the laboratory of the Office of Disease Prevention and Control 5th Ratchaburi province, Thailand from January to December 2015. Drug susceptibility testing (DST) was performed using a BACTEC MGIT 960 system. Furthermore, the genes *katG, inhA, rpoB* and *pncA* were characterized by DNA sequencing.

Results: Of a total of 100 MTB samples which underwent drug susceptibility testing, 42% showed isoniazid (INH) and rifampicin (RIF) resistance, and a further 25% showed INH mono-resistance (25%). The most common gene mutations found using DNA sequencing were *katG*_Ser315Thr (70%), *rpoB*_Ser531leu (81%) and *pncA*_Ile31Thr (84%). The common mutation of *pncA*_Ile31Thr substitution was detected in 26 of 91 (29%) pyrazinamide (PZA) susceptible isolates.

Conclusion: Using DNA sequencing to screen for gene mutations conferring drug resistance may be feasible and use less time than using DST to detect resistance patterns.

Key words: multidrug-resistant tuberculosis; MDR-TB; mutation; DNA sequencing.

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Introduction

Tuberculosis (TB) is a major health problem worldwide. TB is one of the top ten causes of death and the leading cause from a single infectious agent. Millions of people continue to be infected by *Mycobacterium tuberculosis* (MTB) each year. As reported by the Global Tuberculosis Report 2018, MDR-TB was found in 82% of the people who showed resistance to treatment with rifampicin, the most effective first line drug [1]. Thailand is one of the 14 countries of the world with a high rate of HIV-related TB infections and drug-resistant TB, verging on a Thai public health crisis with an estimated 130,000 TB cases (189 cases/100,000 population) in 2009 and 110,000 cases (159 cases/100,000 population) in 2012 [2]. In Thailand, the rate of MDR-TB in 2010 was estimated to be 1.7% among newly diagnosed TB cases and 35% among previously treated cases [3]. Public Health region 5 comprises 8 provinces in the Western region of Thailand, namely: Ratchaburi, Petchaburi, Prachuabkirikhan, Nakhon-Pathom, Kanchanaburi, Suphanburi, Samutsakhon and Samutsongkhram. MDR-TB cases reported by Makarak hospital, Kanchanaburi province, increased from 9.7% in 2007 to 17.2% in 2011 [4] but declined to 8.8% in 2015 [5]. In the Public Health Region 5, new TB cases reported in 2015, 2016 and 2017 were 4731, 5825 and 6140, respectively [6]. Antimicrobial Susceptibility Testing (AST) is the conventional gold standard used to diagnose MDR-TB, but it causes delays in reporting of the resistance pattern when compared to the DNA sequencing method used to determine the mutation of resistance genes. The World Health Organization (WHO) approved the molecular test as an adequate method for MDR-TB diagnosis in 2008. The molecular test is based on the principle of nucleic acid

amplification which allows a prompt and precise detection of isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA) resistance genes in MTB. PZA is the first line drug used for new patients, re-treatment and DR-TB treatment cases, but currently phenotype-based PZA susceptibility testing has not been routinely performed because the acidity of culture medium needed for drug activity resulting in *M. tuberculosis* growth restriction. This study aimed to characterize mutations in *katG*, *inhA*, *rpoB* and *pncA*, genes that are associated with INH, RIF and PZA resistance, respectively, and to determine whether identified mutations confer resistance in MTB strains from the Western region of Thailand.

Methodology

Ethical clearance

This study was approved by the Ethics Committee of the Department of Disease Control, Ministry of Public Health (No.10/60-045).

Bacterial strains

A total of 100 MTB-positive sputum samples taken from patients between January and December 2015 was analyzed. The samples came from previously treated patients suspected of having MDR-TB including: a) re-treatment failure cases with first line drugs, b) first treatment failure patients with first line drugs, c) relapse cases d) loss to follow-up cases, e) patients having had contact with MDR-TB, and d) HIV-positive TB cases. Those MTB isolated from samples were grown in a liquid medium containing Middlebrook 7H9 broth base with fluorescent indicator in the BACTEC MGIT 960 system (Becton, Dickinson Company, Sparks, USA). Isolated strains were confirmed by acid-fast bacilli (Ziehl-Neelsen) stain and SD BIOLINE TB Ag MPT64 rapid test (Standard Diagnostic, Inc., Kyonggi-do, Korea) at the Disease Control Medical Laboratory of Public Health Region 5, Ratchaburi Provinces, Thailand. This Laboratory is the center for TB diagnosis of the Western region of Thailand. Positive *Mycobacterium tuberculosis* strains detected by cultivation in BACTEC MGIT 960 culture fluid were sub-cultured onto Löwenstein-Jensen (LJ) medium (Biomedia Thailand Co., Ltd, Nonthaburi, Thailand) for drug susceptibility testing and extraction of DNA for PCR and sequencing.

First line drug susceptibility testing [7]

First-line drug susceptibility testing for INH and RIF was performed using BACTEC MGIT 960 system. The standard protocol as recommended by the manufacturer for first line drugs was followed for DST by using the BACTEC MGIT 960 method. *Mycobacterium tuberculosis* colonies were scraped from the LJ medium and inoculated into MGIT broth containing 8-10 glass beads for preparing 0.5 McFarland standard suspension. Then, it was diluted 1:5 using a sterile normal saline solution prior to applying 0.5 mL suspension into the MGIT SIRE kit; the final concentrations were 0.1 and 1.0 µg/mL of INH and RIF, respectively. Using predefined algorithms, readings were automatically interpreted by the BACTEC MGIT 960 instrument and reported as either susceptible or resistant. *Mycobacterium tuberculosis* H37Rv strain was used for quality control testing in DST.

Pyrazinamide (*PZA) susceptibility testing*

PZA susceptibility testing was performed using BACTEC MGIT 960 system as recommended by the manufacturer. *Mycobacterium tuberculosis* colonies were scraped from the LJ medium and inoculated into MGIT broth containing 8-10 glass beads for preparing 0.5 McFarland standard suspension. Then it was diluted 1:5 using a sterile normal saline solution prior to applying 0.5 mL suspension into the MGIT PZA kit, final concentration was 100 µg/mL [7]. *Mycobacterium tuberculosis* H37Rv strain was used for quality control.

DNA extraction

One loopful of *M. tuberculosis* colonies harvested from the surface of LJ medium was suspended in TE (Tris-EDTA) buffer and boiled at 100°C for 10 minutes with subsequent precipitation in a 13,000 RPM centrifuge for 3 minutes. The supernatant containing DNA was used as a template for PCR amplification.

PCR and DNA sequencing

Three structural genes (*katG, rpoB, pncA*) and one regulatory region (*inhA* promotor region) were screened for mutations by direct sequencing of each locus. The PCR amplification of *katG*, *inhA*, *rpoB* and *pncA* gene was performed using primers based on previously reported studies (4,5,6) including primers katG-F (5'-AGCTCGTATGGCACCGGAAC-3'), katG-R (5'-AACGGGTCCGGGATGGTG-3'), inhA-F (5'-CCTCGCTGCCCAGAAAGGGA-3'), inhA-R (5'-AGCGCCTTGGCCATCGAAGCA-3'), rpoB-F (5'-TCGCCGCGATCAAGGAGT-3'), rpoB-R (5'-TGCACGTCGCGGACCTCCA-3'), pncA-F (5'-GCGGCGTCATGGACCCTATATC-3') and pncA-R (5'- CTTGCGGCGAGCGCTCCA-3') using Gene Amp PCR System 9700 thermal cycler. The total

volume of PCR was 25 µL that contained 5 µL of boiled DNA template, 20 pmol of each primer and 12.5 µL of AmpliTaq Gold DNA Polymerase mastermix (Applied Biosystems, Foster City, USA). The boiled DNA template of *Mycobacterium tuberculosis* H37Rv was used as a positive control. The thermocycling conditions were one initial denaturation at 95°C for 10 minutes, followed by 30 cycles involving 95°C for 60 seconds, 61°C for 30 seconds and 72°C for 30 seconds and then the final extension 72ºC for 10 minutes. The amplified products of *katG* (200 bp), *inhA* (1,331 bp), *rpoB* (157 bp), *pncA* (696 bp) were separated in a 1% agarose gel and visualized using a UV transluminator. The PCR products were purified using hydrolytic enzyme Exonuclease I and Shrimp Alkaline Phosphatase (New England Biolab Inc, Ipswich, USA), and then subjected to Sanger sequencing by ABI 3130 genetic analyzer (Applied Biosystems, Foster City, USA) with Big Dye terminator v 3.1 sequencing kit, using the same primers as used for amplification [8]. The obtained sequence was analyzed with SeqScape version 3.1 (Applied Biosystems, Foster City, USA) sequencing analysis software, comparing the multiple sequence alignments with the wild-type sequences of *katG*, *inhA* promotor, *rpoB,* and *pncA* with accession numbers X68081.1, U41388.1, U12205.1 and AL123456.3 respectively.

Results

Drug susceptibility testing

Among the 100 *Mycobacterium tuberculosis* strains, 81 strains were drug-resistant, each of which exhibited resistance to one or more of the following anti-tuberculosis agents: INH, RIF and PZA. Phenotypic analysis of 81 resistant strains revealed that 31 (38%) were resistant to one antibiotic, whereas 46 strains (57%) and four strains (4.9%) were resistant to two and three test drugs, respectively (Table 1). The drug resistant TB (DR-TB) rates for individual drugs - INH, RIF, and PZA - were found to be 74/81 (91%), 52/81 (64%) and 9/81 (11%), respectively. Of the 46 identified MDR-TB strains were 42/81 (52 %) showed resistance to INH and RIF and 4/81 (4.9%) showed resistance to INH, RIF and PZA (Table 1). Furthermore, 28/81 strains exhibited resistance to INH but not to RIF, while 6/81 (7.4%) strains were resistant to RIF but not to INH. There was one strain which exhibited resistance to PZA without involving either INH or RIF. Contrary to the high rates of resistance to RIF and INH, 72 (89%) of 81 strains remained susceptible to PZA.

DNA sequencing

The results of nucleotide mutation analysis of clinical strains resistant (81 isolates) and susceptible (19 isolates) to tested drugs are as follows. Among the four genes studied, *katG* and *rpoB* were found to comprise the largest range of mutation genotypes, with a total of 52 and 41 mutation patterns identified, respectively. The most common mutation of the *katG* gene fragments was found to be *katG*_Ser315Thr (52 strains), whereas the mutations patterns of the *inhA*, *rpoB* and *pncA* gene fragments were characterized by a single nucleotide change at *inhA*_-15 (C>T) (15 strains), *rpoB*_Ser531leu (41 strains) and *pncA*_Ile31Thr (26 strains), respectively.

INH resistance gene mutation

In this study, the *katG* and *inhA* genes were analyzed for mutations conferring resistance to INH (Table 2). Only one pattern of *katG* mutation, namely *katG*_Ser315Thr, was identified in this study. However, two mutation patterns of *inhA* gene were identified, which were *inhA*_-15 (C>T) and *inhA*_3Gly (silent mutation). Of a total of 74 INH-resistant strains 52 (70%) strains were found to carry *katG*_Ser315Thr, 15 (20%) strains were found to carry *inhA*_-15 (C>T) and 1 (1.4%) strain was found to carry *inhA*_3Gly (silent mutation). Of the 74 (91%) INH-resistant strains 63 (85%), three (4.0%) and eight (11%) strains carried a single mutation, a double mutation and no mutation in the *katG* and *inhA* genes, respectively. It was found that

Table 1. Drug susceptibility testing for INH, RIF and PZA by BACTEC™ MGIT™ 960 System (n = 100).

DST	DST pattern	Number) %(
Resistant (n = 81)	INH resistance	25 (30.9)
	RIF resistance	5 (6.2)
	PZA resistance	1 (1.2)
	INH+RIF resistance	42 (51.9)
	INH+PZA resistance	3 (3.7)
	RIF+PZA resistance	1 (1.2)
	INH+RIF+PZA resistance	4 (4.9)
Susceptible (n = 19)	INH+RIF+PZA susceptible	19 (100)

DST = drug susceptibility testing; INH = isoniazid; RIF = rifampicin; PZA = pyrazinamide.

the INH-resistant strains with a single mutation comprised 49 (66%) strains exhibiting *katG*_Ser315Thr and 14 (19%) strains exhibiting *inhA*_-15 (C>T). Among INH-resistant strains with a double mutation, two strains exhibited both *katG*_Ser315Thr and *inhA*_3Gly (silent mutation) and one strain exhibited both *katG*_Ser315Thr and *inhA*_-15 (C>T). Two of the INH-susceptible strains carried the mutation of *inhA*_3Gly (silent mutation). Mutations in the *katG* codon 315 and/or *inhA* promotor region were not detected in all susceptible strains. Therefore, if the mutations of *katG*_Ser315Thr and *inhA*_-15 (C>T) were considered to confer resistance to INH, the

Table 2. Characterization of katG, inhA, rpoB and pncA genes (n = 100).

DST pattern	n	genotype			
		katG	*inhA*	*rpoB*	*pncA*
Monodrug-resistance					
INH resistance (n = 25)	1	Ser315 Thr	3Gly (Silent mutation)	0	0
	1	Ser315 Thr	-15 (C>T)	0	0
	6	Ser315 Thr	0	0	Ile31Thr
	8	Ser315 Thr	0	0	0
	1	0	-15 (C>T)	0	Ile31Thr
	5	0	-15 (C>T)	0	0
	3	0	0	0	0
RIF resistance (n = 5)	1	0	0	His526Tyr	Ile31Thr
	1	0	0	Ser531Leu	Ile31Thr
	3	0	0	Ser531Leu	0
PZA resistance (n = 1)	1	0	0	0	0
Multidrug-resistance					
INH resistance + RIF resistance (n = 42)	1	Ser315 Thr	3Gly (Silent mutation)	Ser531Leu	Ile31Thr
	1	Ser315 Thr	0	Asp516Val	0
	1	Ser315 Thr	0	His526Asn	Ile31Thr
	1	Ser315 Thr	0	His526Asp	0
	1	Ser315 Thr	0	His526Tyr	0
	10	Ser315 Thr	0	Ser531Leu	Ile31Thr
	14	Ser315 Thr	0	Ser531Leu	0
	1	Ser315 Thr	0	Ser531Trp	0
	1	Ser315 Thr	0	Leu533 Pro	0
	1	0	-15 (C>T)	His526Tyr	0
	3	0	-15 (C>T)	Ser531Leu	Ile31Thr
	3	0	-15 (C>T)	Ser531Leu	0
	1	0	0	His526Tyr	0
	1	0	0	Ser531Leu	Ile31Thr
	2	0	0	Ser531Leu	0
INH resistance + RIF resistance + PZA resistance (n = 4)	1	Ser315 Thr	0	Ser531Leu	Val7Gly
	1	Ser315 Thr	0	Gln513Lys	Thr142Ala
	1	Ser315 Thr	0	His526Pro	0
	1	0	-15 (C>T)	Ser531Leu	0
Polydrug-resistance					
INH resistance + PZA resistance (n = 3)	1	Ser315 Thr	0	0	Ile90Ser
	1	Ser315 Thr	0	0	Ser59Tyr
	1	0	0	0	0
RIF resistance + PZA resistance (n = 1)	1	0	3Gly (silent mutation)	Ser531Leu	122Gly (silent mutation)
Susceptible					
INH susceptible + RIF susceptible +PZA susceptible (n = 19)	1	0	3Gly (silent mutation)	0	0
	1	0	0	0	Ile31Thr
	17	0	0	0	0

DST = drug susceptibility testing; INH = isoniazid; RIF = rifampicin; PZA = pyrazinamide.

sensitivity and specificity were 89% and 100%, respectively (Table 3).

RIF resistance gene mutation

A total of 52 RIF-resistant strains carried a mutation in *rpoB*. Mutations in this gene were not found in any of the RIF-susceptible strains. The mutation patterns were detected in *rpoB* including: Ser531Leu (42/52), His526Tyr (4/52), His526Asn (1/52), His526Asp (1/52), His526Pro (1/52), Ser531Trp (1/52), Asp516Val (1/52), Leu533Pro (1/52) and Gln513Lys (1/52) (Table 2). The sensitivity and specificity of each gene mutation are indicated in Table 3.

PZA resistance gene mutation

A total of 31 strains carried a mutation in *pncA*. These mutations consisted of *pncA*_Ile31Thr (26/31), Val7Gly (1/31), Thr142Ala (1/31), Ile90Ser (1/31), Ser59Tyr (1/31), and 122Gly (silent mutation) (1/31). The sensitivity and specificity of each gene mutation associated with PZA resistance is indicated in Table 3. Although nine strains were resistant to PZA by DST, only five of these strains carried a *pncA* gene mutation including Val7Gly (1/9), Thr142Ala (1/9), Ile90Ser (1/9), Ser59Tyr (1/9), and 122Gly (silent mutation) (1/9) but four of these strains shown no mutation. The remaining 26 strains carrying the *pncA*_Ile31Thr mutation were susceptible to PZA. This suggests that the *pncA*_Ile31Thr mutation does not confer PZA resistance.

Discussion

Although the joined effort of various countries has established strategies to stop or reduce MTB infection each year, the trend of MTB resistance has increased despite the use of vaccine or several antibiotics. This study conducted an analysis of sputum samples from patients with presumed MDR-TB and identified a high percentage of MDR-TB strains. One explanation may be close contact with known TB and MDR-TB patients, an association that was reported in several studies [9-12]. However, other risk factors associated with MDR-TB may be age, previous history of TB, HIV co-infection and alcohol consumption but not TB contact as reported elsewhere [13]. No matter what the actual cause is, early detection for MTB resistance is essential to stop the spread of this infection. Genetic testing of drug resistance is an alternative to conventional susceptibility testing due to its ability to provide rapid and accurate results. Gene mutations associated with INH, RIF and PZA were characterized in this study. Not all mutations detected were found to be associated with resistance. For INH, *inhA*, *katG* and the promoter region -15 (C>T) are useful for determining resistance. Although many reports revealed a variety of mutations associated with INH resistance [14-16], the analysis of

Table 3. Sensitivity, specificity, PPV and NPV of the *katG, inhA, rpoB* and *pncA* mutations for predicting the INH, RIF and PZA resistance.

Mutated codon	No. of isolates	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
katG					
315 (Serine>Threonine)	52	70.27	100	100	54.16
inhA					
-15(C>T)	15	20.27	100	100	30.58
katG* and *inhA					
315 (Serine>Threonine) and -15(C>T)	66	89.19	100	100	76.47
rpoB					
513 (Glutamine>Lysine)	1	1.92	100	100	48.48
516 (Aspartate>Valine)	1	1.92	100	100	48.48
526 (Histidine>Asparagine)	1	1.92	100	100	48.48
526 (Histidine>Aspartate)	1	1.92	100	100	48.48
526 (Histidine>Proline)	1	1.92	100	100	48.48
526 (Histidine>Tyrosine)	4	7.69	100	100	50.00
531 (Serine>Leucine)	41	78.84	100	100	81.36
531 (Serine>Tryptophan)	1	1.92	100	100	48.48
533 (Leucine>Proline)	1	1.92	100	100	48.48
pncA					
7 (Valine>Glycine)	1	11.11	100	100	91.92
59 (Serine>Tyrosine)	1	11.11	100	100	91.92
90 (Isoleucine>Serine)	1	11.11	100	100	91.92
122 Glycine>(silent mutation)	1	11.11	100	100	91.92
142 (Threonine>Alanine)	1	11.11	100	100	91.92

PPV = positive predictive value, NPV = negative predictive value.

the associated gene in strains isolated from the Western region of Thailand found only one mutation of *katG* at codon 315 and two mutations of *inhA* including the promoter region -15 (C>T) and *inhA*_3Gly (silent mutation). Although the silent mutation of *inhA* codon 3 was found in two INH-resistant strains, two INH-susceptible strains carried the mutation of *inhA*_3Gly (silent mutation). Consistent with Luo *et al*. (2010), two INH-resistant strains carried *inhA*_3Gly (silent mutation) and *katG*_Ser315Thr [17]. In addition, the study of Ong *et al*. [18] used 23 drug resistant *M. tuberculosis* reference strains to validate high resolution melting (HRM) assay. Reference strain number IR27 also carried *inhA*_3Gly (silent mutation) and *ahpC* at region -5 (G>A). There is no report of *inhA*_3Gly (Silent mutation) found in INH -susceptible strains as indicated in this study. Therefore, the only single mutation of *inhA*_3Gly (silent mutation) may not be associated with INH resistance. However, the mutation in *katG*_Ser315Thr may be a major locus because these mutations decrease INH activation without abolishing catalase-peroxidase activity [19]. Of the 74 INH-resistant strains, 66 (89%) had mutations either in *katG* or *inhA* of which 52 (70%) strains carried the mutation *katG*_Ser315Thr followed by 14 (19%) strains with the mutation *inhA*_-15 (C>T) but not *katG*_Ser315Thr. These results suggests that the mutations in codon 315 of the *katG* gene and in the promotor region of *inhA* are also the most common mutations involved in INH resistance [20], depending on the geographic region studied. Similar results with high rates of *katG* mutations were found in many countries including Egypt (92%), the central area of Thailand (79%), Vietnam (71%), China (86%). Myanmar (64%) and Malaysia (70%) [21-25]. Several reports demonstrated that mutations in the *inhA* promoter region appeared with low frequency. From a previous report from the USA, Thailand and Korea, the frequency of -15 (C>T) point mutations is 39%, 14% and 25%, respectively [22,26,27]. Therefore, eight strains from this study without the mutation in *katG*_Ser315Thr *inhA*_and -15 (C>T) may carry mutations located in other *katG* or *inhA* loci [28,29] or other associated genes such as *ahpC* and *ndh* gene [30]. However, the mutations *katG*_Ser315Thr and *inhA*_-15 (C>T), both of which showed high sensitivity (89%) and high specificity (100%), could be used as indicators for detecting INH resistance. Similarly to the molecular technique, the line probe assay (LPA), recommended by the WHO, the accuracy of LPA was evaluated by detecting INH resistance and by comparing with both DNA sequencing of the *inhA* gene promotor and the *katG* gene as well as DST. Bivariate analysis revealed a pooled sensitivity of 85% (95% CI: 80-88.6) and a pool specificity of 99.5% (95% CI: 99.1-99.8) [31]. For RIF, *rpoB* gene is the active region for determining its resistance. All locations of *rpoB* mutations identified in this study were associated with RIF resistance. Here, the most common mutation involved in RIF resistance was a missense mutation in codon Ser531Leu of *rpoB* (Table 3). These results were similar to those of previous studies [26,32,33]. The frequency of this mutation in this study was 79% (41/52) whereas other studies reported frequencies of 27% [24], 64% [29] and 60% [30]. The second most common mutation was His526Tyr (7.8%). In addition, His526Asn, His526Asp, His526Pro, Ser531Trp, Gln513Lys, Asp516Val and Leu533Pro were detected associated with resistance. As found in previous studies, certain *rpoB* mutations indicate different levels of tolerance to RIF [34,35]. The amino acid change in each mutation position determines the level of tolerance. Mutations Ser531Leu, Ser531Trp, His526Tyr, His526Asp, His526Pro, and Gln513Lys confer a high tolerance to RIF. (MIC ≥ 100 μg/mL), whereas Asp516Val, His526Asn and Leu533Pro are associated with moderate (MIC ≥ 20 to < 100 μg/mL) or low(MIC >1 to < 20 μg/mL) [34-42]. For this reason, all *rpoB* missense mutations are related to RIF resistance resulting in 100% sensitivity and specificity. This resistance result is slightly more accurate than those obtained with the LPA used to detect RIF resistance compared with a reference standard included both DNA sequencing of the *rpoB* gene and phenotypic culture-based DST. Bivariate analysis revealed a pooled sensitivity of 95.3% (95% CI: 93.4-96.6) and a pool specificity of 99.5% (95% CI: 98.6-99.8) [31]. Up to the present, sequence analysis of the *rpoB*, *katG* and *inhA* gene promoters is useful to detect RIF/INH -resistant *M. tuberculosis* isolates. On the other hand, the genetic variation of *pncA*_Ile31Thr was most frequently detected in this study, but was not associated with PZA resistance. This mutation at codon 31 of *pncA* was previously reported in the central region of Thailand [43,44]. The *pncA* mutation associated with PZA resistance was detected in five out of nine strains (56%) each in a different position, including Val7Gly, Thr142Ala, Ile90Ser, Ser59Tyr and 122Gly (silent mutation). The mutations in *pncA* including Val7Gly, Thr142Ala, Ile90Ser, and Ser59Tyr were reported in previous studies [45-48], whereas the novel silent mutation Gly122Gly was only characterized in this study. Some resistant strains were reported with a silent mutation at codon 65 (Ser65Ser) that was not associated

with PZA resistance [49,50]. However, Lai *et al.* (2018), reported *rpoB*474 and *gyrA86/126* silent mutations in methicillin-resistant Staphylococcus isolates [51]. Investigating silent mutations is attracting great interest [52,53]. Silent mutations can affect gene expression. The study of Patel *et al*. (2019) generated several silent mutants of the ω - subunit of *Escherichia coli* RNA polymerase. Not all silent mutations affected the structure, some reduced the expression of the constitutively expressed gene. When reconstituted with the silent mutant ω7 the RNA polymerase was transcriptionally inactive [54]. Future studies should investigate how the silent mutation Gly122Gly detected in this study is involved in PZA resistance. Another four (44%) PZA-resistant strains did not show any nucleotide change suggesting that the mechanism of resistance was not conferred by mutations in *pncA*, but could involve decreasing the drug delivery into the bacterial cell or increasing the efflux pump. Resistance may also involve mutations in other genes that were not tested here such as *rpsA* [55]. Based on these results, sequence analysis of *pncA* may not be appropriate for detecting PZA resistance.

Conclusion

In conclusion, there are different kinds of mutation occurring at various target loci relating to INH, RIF and PZA resistant clinical strains. There were 76 of 81 resistant MTB strains (94%) which could be detected by using the 4 target genes *rpoB*, *katG*, *inhA* and *pncA*. The sequence analysis of the *rpoB*, *katG* and *inhA* gene promoter region is useful to detect RIF/INH resistant *M. tuberculosis* isolates. It was clear that sequencing of the *pncA* gene alone was not sufficient to predict PZA resistance. Screening of mutations conferring INH and RIF resistance using DNA sequencing may be feasible and may use less time to detect resistance patterns compared to DST and may also be used to detect novel mutations in resistance genes.

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