

Analyzing *dprE1* gene variations in drug-resistant *Mycobacterium tuberculosis* isolates from Northern Thailand

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ARTICLE INFO

Article history:

Received 23 August 2024

Accepted as revised 18 September 2024

Available online 23 September 2024

Keywords:

Mycobacterium tuberculosis, drug resistance, *DprE1*, mutation.

ABSTRACT

Background: DprE1 has been discovered as a highly promising candidate for eradicating multidrug-resistant tuberculosis (MDR-TB) due to its localization and crucial role in cell wall synthesis. Several DprE1 inhibitors in clinical trials target different binding sites on the DprE1 protein. However, mutations in these binding sites can result in resistance to these inhibitors. Nonetheless, only a few reports exist on the sequence analysis of the *dprE1* gene in drug-resistant clinical isolates, especially in Thailand, a country with a high TB burden.

Objective: To investigate the genetic variations in the *dprE1* gene among drug-resistant clinical *Mycobacterium tuberculosis* (Mtb) isolates from Northern Thailand and to examine mutations known to confer resistance to specific inhibitors.

Materials and methods: Drug resistance profiles of 19 clinical Mtb isolates from Northern Thailand—11 multidrug-resistant Mtb, 2 isoniazid-resistant Mtb, and 6 rifampicin-resistant Mtb—were assessed using the agar proportion method and Sanger sequencing. New PCR and sequencing primers were designed to cover the entire length of the *dprE1* gene. Mutation profiles were detected by performing DNA alignment against the reference strain, *M. tuberculosis* H37Rv, using BioEdit Sequence Alignment version 7.2.5. The distribution of these mutations was assessed through the Basic Local Alignment Search Tool (BLAST) server. Visual representation of amino acid mutation regions in DprE1 was generated using the PyMOL 3.0.

Results: The mutation at codon 387 associated with benzothiazinone (BTZ) compounds was not observed in the *dprE1* gene of any clinical isolates. The most common mutation profile, C→T at codon 459, was found in 73.68% of clinical isolates. However, this is a silent mutation and probably indicates genetic polymorphism. Non-synonymous substitutions in the *dprE1* gene were detected: A356T in one INH-R isolate and V400I in two RIF-R isolates. BLAST analysis revealed that the A356T mutation in the INH-R isolates had a 100% match with sixteen Mtb strains from other regions. Interestingly, the V400I mutation in the RIF-R isolates had not been reported, making this the first documentation of such a finding.

Conclusion: Although no mutations directly linked to resistance against current DprE1 inhibitors were found, mutations in the DprE1 binding sites could affect the efficacy of these inhibitors. This study highlights the significance of mono-drug-resistant strains that acquire *dprE1* mutations, potentially complicating future TB treatment targeting DprE1.

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doi: 10.12982/JAMS.2025.008

E-ISSN: 2539-6056

Introduction

Tuberculosis (TB) remains one of the global burden diseases, with approximately 1.5 million deaths and more than 10 million new cases yearly.¹ The occurrence of new cases is associated with the reactivation of latent TB infection, possibly due to poverty, malnutrition, and its

coexistence with HIV/AIDS.² Despite the significant efficacy of existing anti-TB drugs, the non-compliance by patients and the complex defense mechanisms of *Mycobacterium tuberculosis* (Mtb) can lead to the development of drug resistance.^{3,4} Currently, eradication of multidrug-resistant TB (MDR-TB), which is expressed as resistance to two first-line antibiotics, isoniazid (INH) and rifampicin (RIF), is the main challenge in TB control.⁵ Failure to effectively treat MDR-TB can lead to the emergence of extensively drug-resistant TB (XDR-TB), highlighting the need to explore new molecular targets for treatment.

The mycobacterial cell wall is unique due to its lipid-rich, complex structure, which protects Mtb from the harsh environment of the host and its antibiotic effects.⁶ Consequently, cell wall synthesis plays an essential process in Mtb survival, and genes involved in this

pathway become the target of the first-line drugs such as isoniazid and ethambutol, which inhibit mycolic acid and arabinogalactan synthesis of the cell wall of Mtb, respectively.⁷ The flavoenzyme Decaprenylphosphoryl- β -D-ribose-2'-epimerase oxidase (DprE1, Rv-3790) is an essential enzyme involved in the synthesis of the arabin components of lipoarabinomannan (LAM) and arabinogalactan (AG), both of which are important for cell wall integrity and immunomodulatory role of Mtb.^{8,9} DprE2 and DprE1 facilitate the two-step epimerization of decaprenyl-phospho-ribose (DPR). Initially, DprE1 catalyzes the conversion of DPR into decaprenyl-phospho-20-keto-D-arabinose (DPX), with a concurrent reduction of the FAD cofactor to FADH₂. Subsequently, DPX is reduced to DPA by DprE2, serving as the exclusive precursor for the synthesis of AG and LAM (Figure 1).¹⁰

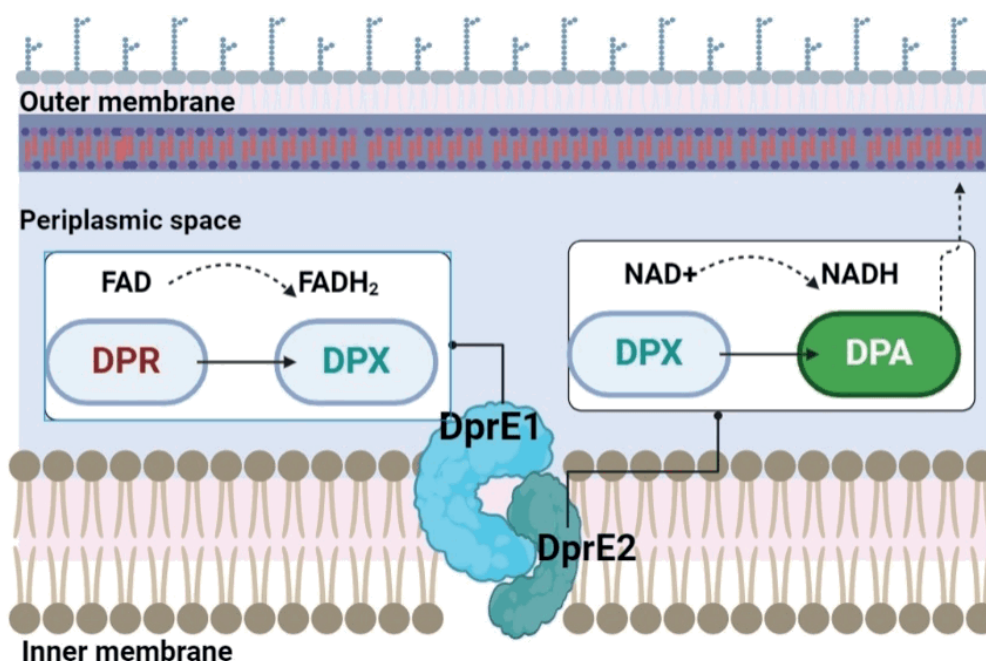


Figure 1. The role of DprE1 and DprE2 in cell wall components construction. Created with Biorender (biorender.com).

DprE1 is often described as a “magic drug target” because it is highly conserved in Mtb and the epimerization process it mediates occurs in the periplasmic space, which makes it more accessible to inhibitors.^{8,11} DprE1 was initially identified as a target of benzothiazinones (BTZ), which irreversibly inhibit the enzyme by forming a covalent adduct with the amino acid Cysteine at codon 387 (Cys387).¹² In contrast, some noncovalent inhibitors, such as TBA-7371, act as competitive inhibitors.¹⁰ Both covalent and non-covalent inhibitors targeting DprE1, including Macozinone, BTZ-043, and TBA-7371, are currently undergoing clinical trials.¹³ However, it remains crucial to identify any mutations in clinical isolates of Mtb because, unlike Mtb, *M. avium* and *M. aurum* have serine or alanine at position 387, which leads to natural resistance to BTZ.^{12,14}

Researchers monitored potential BTZ resistance in a study by analyzing the DprE1 sequences of Mtb clinical isolates from four European Hospitals. They found all

isolates were susceptible to BTZ and lacked mutations in the Cys387 region. This study concluded that mutations associated with BTZ resistance are not present in the currently circulating strains of Mtb.¹⁵ However, as most strains in this study were from drug-susceptible (DS) TB cases, further research is needed to analyze the genetic variation of the DprE1 in MDR-TB clinical isolates. This is essential because drugs targeting DprE1 are critical for treating MDR-TB, and comprehensive information on mutations in DprE1 from drug-resistant clinical isolates in Northern Thailand remains limited.

Therefore, the present study aims to investigate the genetic diversity and identify mutations of the *dprE1* gene in MDR-TB and mono-resistance to isoniazid and rifampicin isolates from Northern Thailand. The findings would reveal patterns of mutation in the *dprE1* gene. This may contribute to developing new drugs that avoid common mutation sites for interaction, thereby improving the treatment of drug-resistant TB.

Materials and methods

Mycobacterial strains

The Mtb strains exhibiting diverse drug resistance profiles (11 MDR, 2 INH-R, and 6 RIF-R) were kindly provided by the Office of Disease Prevention and Control 1 (Chiang Mai, Thailand). These strains were isolated from TB patients in Northern Thailand between 2015 and 2019. Lowenstein-Jensen (L-J) medium (Biomedica, Thailand) was used to culture all Mtb clinical isolates at 37 °C for 1 month.

Determination of drug-resistant profiles

The agar proportion method was used to perform standard drug susceptibility testing (DST) of Mtb isolates against first-line anti-TB drugs. As previously described, an isolate was classified as resistant if more than 1% of the colony grew on Middlebrook 7H10 agar plates when exposed to the critical drug concentrations: 0.2 µg/mL for isoniazid (INH), 1.0 µg/mL for rifampicin (RIF), 2.0 µg/mL for streptomycin (SM), and 5.0 µg/mL for ethambutol (EMB).¹⁶ Additionally, the mutation profiles of each isolate were confirmed by DNA sequencing. This included identifying *rpoB* mutations associated with rifampicin resistance (RIF-R) and mutations in *katG* and *inhA* genes linked to isoniazid resistance (INH-R).

Design of sequencing primers

Using the Primer-BLAST program (NCBI), primers were designed to cover the entire length of the *dprE1* gene (1385 bp) based on the nucleotide sequence (4235779-4237164) of *dprE1* gene in *M. tuberculosis* H37Rv, complete genome (CP003248.2). The forward primer, *dprE1_F*, is 5'-GTCGGACTCAACCACCTCTG-3' and reverse primer, *dprE1_R*, is 5'-GATCAAAAAGCCGAAGCGCC-3'.

DNA extraction and PCR amplification of the *dprE1* gene

The genomic DNA of each Mtb clinical isolate was extracted according to the manufacturer's instructions

using the Anyplex™ II MTB/MDR Detection kit (see gene, the Republic of South Korea). The extracted DNA was kept at -20 °C before use. PCR amplification of the *dprE1* gene was performed using the newly designed primers. The 50-µL PCR reaction included KOD One™ PCR Master Mix (Toyobo, Japan), 0.3 µM of primers, and 100 ng of DNA template. The PCR conditions were carried out as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 68 °C for 15 sec, and extension at 72 °C for 10 sec, followed by 72 °C for 2 min as a final extension. The resulting DNA fragments were examined by electrophoresis on a 1.5% (w/v) agarose gel at 80 volts and visualized using ethidium bromide staining. Before Sanger sequencing, the *dprE1* amplicons were purified using the NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Germany). The quantity and purity of the amplicons were measured by UV spectrophotometer (Biotech Epoch™).

Analysis of the *dprE1* gene sequences

Sanger sequencing was performed after DNA amplification to study the sequence of *dprE1* in different clinical isolates. Initially, the purified DNA was mixed with the BigDye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) and primers, then processed on the ABI Prism 3730 XL automated sequencer. Then, mutation profiles were investigated by performing DNA alignment of clinical isolates with *M. tuberculosis* H37Rv (GenBank: CP003248.2) as the reference using BioEdit Sequence Alignment version 7.2.5 and the Basic Local Alignment Search Tool (BLAST) server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Structural illustrations of amino acid mutations of DprE1 were generated using PyMOL 3.0 after retrieving the hexagonal crystal structure of *Mycobacterium tuberculosis* *dprE1* (4FEH) from the Protein Data Bank (PDB) (Figure 2).

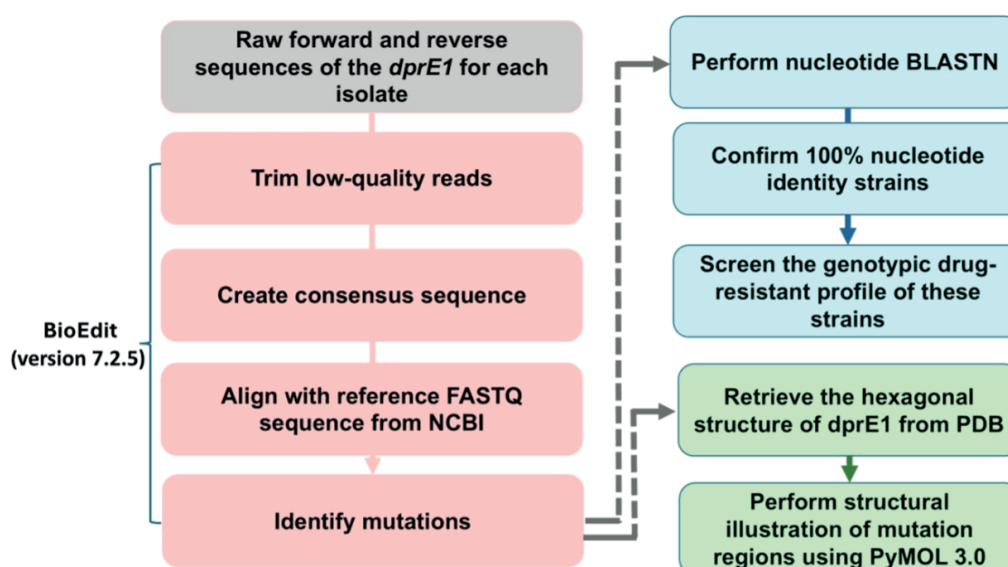


Figure 2. Overview of the sequence analysis workflow.

Result

The genotypic-phenotypic correlation of Mtb clinical isolates

In the agar proportion method, the resistance of Mtb isolates to at least INH and RIF were classified as MDR, isolates resistant only to INH were identified as INH-R, and those resistant to RIF for RIF-R (Table 1, 2, 3). Analysis of the mutation profiles of each isolate revealed that two

MDR isolates (MDR 56 and 57) lacked mutations in INH resistance-associated genes (*inhA* and *katG*), and one RIF-R isolate (51108) was absent of mutations in RIF resistance-associated genes (*rpoB*) (Table 1, 2, 3). However, mutation profiles of the remaining isolates correlated with their DST results.

Table 1. DST and mutation profiles of 11 MDR clinical isolates.

Code strains	DST				Mutation profiles		
	RIF	INH	EMB	SM	<i>rpoB</i>	<i>katG</i>	<i>inhA</i>
MD47	R	R	S	S	D516V	No	T-9C
MD45	R	R	S	S	S522L	S315T	G-17T
MD56	R	R	S	S	H526Y	No	No
MD57	R	R	S	S	S531L	No	No
MD65	R	R	S	R	S531L	S315T	No
sub05	R	R	R	R	S531L	S315T	No
Og43	R	R	S	R	H526Y	S315T	No
Sub10	R	R	S	R	S531L	S315T	No
49030	R	R	S	S	S531L	S315T	No
49753	R	R	S	S	S531L	S315T	No
51268	R	R	S	S	H526D	S315T	No

Note: D516C: substitution of cysteine for aspartic acid of *rpoB* at codon 516, S522L: substitution of leucine for serine of *rpoB* at codon 522, H526Y: substitution of tyrosine for histidine of *rpoB* at codon 526, S531L: substitution of leucine for serine of *rpoB* at codon 531, H526D: substitution of aspartic acid for histidine of *rpoB* at codon 526, S315T: substitution of threonine for serine of *KatG* at codon 315, T-9C: nucleotide change from T to C at *inhA* promoter region (-9), G-17T: nucleotide changes form G to T at *inhA* promoter region (-17), No: no mutation, DST: drug susceptibility testing, R: resistant, S: sensitive, MDR: multi-drug resistance, RIF: Rifampicin, INH: Isoniazid, EMB: Ethambutol, SM: Streptomycin.

Table 2. DST and mutation profiles of 2 INH-R clinical isolates.

Code strains	DST				Mutation profiles		
	RIF	INH	EMB	SM	<i>rpoB</i>	<i>katG</i>	<i>inhA</i>
52200	S	R	S	S	No	No	C-15T
50886	S	R	S	S	No	No	C-15T

Note: C-15T: nucleotide change from C to T at *inhA* promoter region (-15), No: no mutation, DST: drug susceptibility testing, R: resistant, S: sensitive, INH-R: Isoniazid resistance, RIF: Rifampicin, INH: Isoniazid, EMB: Ethambutol, SM: Streptomycin.

Table 3. DST and mutation profiles of 6 RIF-R clinical isolates.

Code strains	DST				Mutation profiles		
	RIF	INH	EMB	SM	<i>rpoB</i>	<i>katG</i>	<i>inhA</i>
R41	R	S	S	S	L511P	No	No
R42	R	S	S	S	L511P	No	No
49698	R	S	S	S	S531L	No	No
49505	R	S	S	S	H526Y	No	No
50071	R	S	S	S	H526Y	No	No
51108	R	S	S	S	No	No	No

Note: L511P: substitution of proline for leucine of *rpoB* at codon 511, S531L: substitution of leucine for serine of *rpoB* at codon 531, H526Y: substitution of tyrosine for histidine of *rpoB* at codon 526. No: no mutation, DST: drug susceptibility testing, R: resistant, S: sensitive, RIF-R: Rifampicin resistance, RIF: Rifampicin, INH: Isoniazid, EMB: Ethambutol, SM: Streptomycin.

PCR amplification of the *dprE1* gene by the specific primers

The specificity of the PCR product of each isolate was demonstrated by agarose gel electrophoresis. The presence of a single band at the expected product size validated the specificity of the primer as presented (Figure 3).

Predominant nucleotide mutation in MDR strains

Sequence analysis of the *dprE1* gene in 19 Mtb isolates was conducted. Among the 11 multidrug-resistant (MDR) isolates, alignment with the reference sequence revealed a nucleotide mutation at codon 459 (C→T) in 9 out of 11 (81.82%) isolates. In contrast, the remaining two MDR

isolates showed no mutations (Figure 4). The mutation at codon 459 led to a synonymous substitution (a mutation that does not change the amino acid sequence) at amino acid position 153. This finding aligns with previous research, where the C→T mutation at this codon was observed in 96 samples from the NCBI database, predominantly from China (13%) and India (28%), especially in drug-resistant Mtb strains.¹⁷ The recurrence of this mutation across diverse geographic regions suggests that it could be part of a broader adaptive response to drug pressure in the global epidemiology of drug-resistant Mtb.

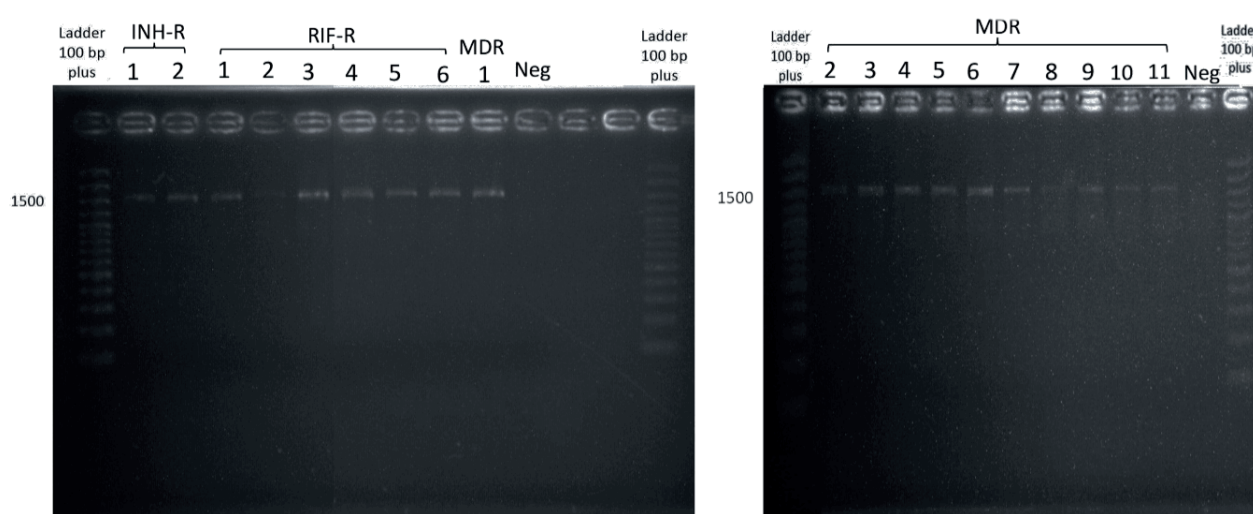


Figure 3. Gel electrophoresis of specific PCR products from 19 clinical isolates.

Sequence alignment of MDR isolates

	450	460	470	480
H37Rv reference	CAGCATGGACCTGCTGACCGCGAGATCCGTCATCTCA			
MD45	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
MD47	CAGCATGGACCTGCTGACCGCGAGATCCGTCATCTCA			
MD56	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
MD57	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
MD65	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
Sub05	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
Sub10	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
Og43	CAGCATGGACCTGCTGACCGCGAGATCCGTCATCTCA			
49030	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
49753	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			
51268	CAGCATGGACCTGCTGACCTGCCGACGGCGAGATCCGTCATCTCA			

*

Figure 4. Sequence alignment of *dprE1* in 11 MDR clinical isolates against the reference sequence from NCBI - *Mycobacterium tuberculosis* H37Rv, CP003248.2 (H37Rv reference). The position of the nucleotide mutation is indicated with *.

Unique non-synonymous substitution in INH-R and RIF-R isolates

One INH-R isolate exhibited the same C→T nucleotide mutation at codon 459 in the mono-resistant isolates, resulting in the same amino acid sequence observed in MDR strains. Another INH-R isolate displayed a G→A mutation at nucleotide position 1066 in the *dprE1* gene, leading to an amino acid substitution of threonine (T) for

alanine (A) at codon 356 (A356T) (Figure 5). BLAST analysis of these sequences against the NCBI database revealed a 100% nucleotide identity of the *dprE1* gene sequences of the 16 DS-Mtb and DR-Mtb strains from various countries in the different geographic regions (Figure 6). Genotypic drug-resistant profiles of all 16 sequence entries were analyzed by screening for mutation in *rpoB*, the *inhA* regulatory region, *inhA*, and *katG* as reported in Table 4.

Sequence alignment of INH-R isolates

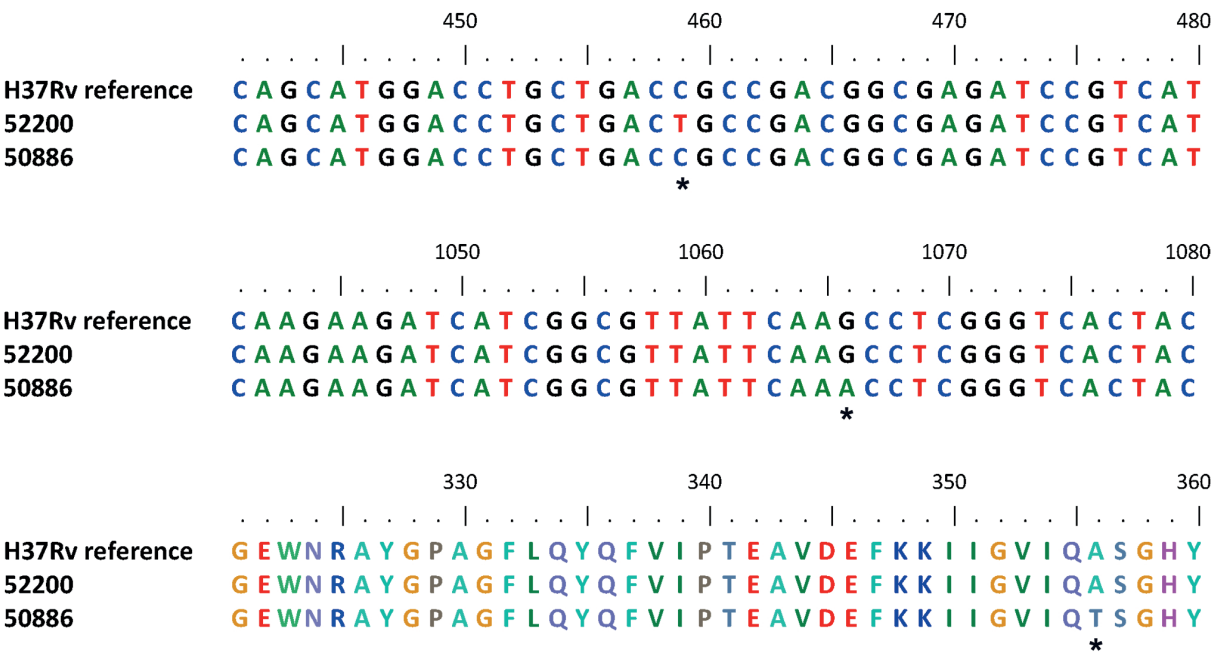


Figure 5. Sequence alignment of *dprE1* in 2 INH-R clinical isolates against the reference sequence from NCBI - *Mycobacterium tuberculosis* H37Rv, CP003248.2 (H37Rv reference). The position of the nucleotide mutation and amino acid substitution are indicated with *.

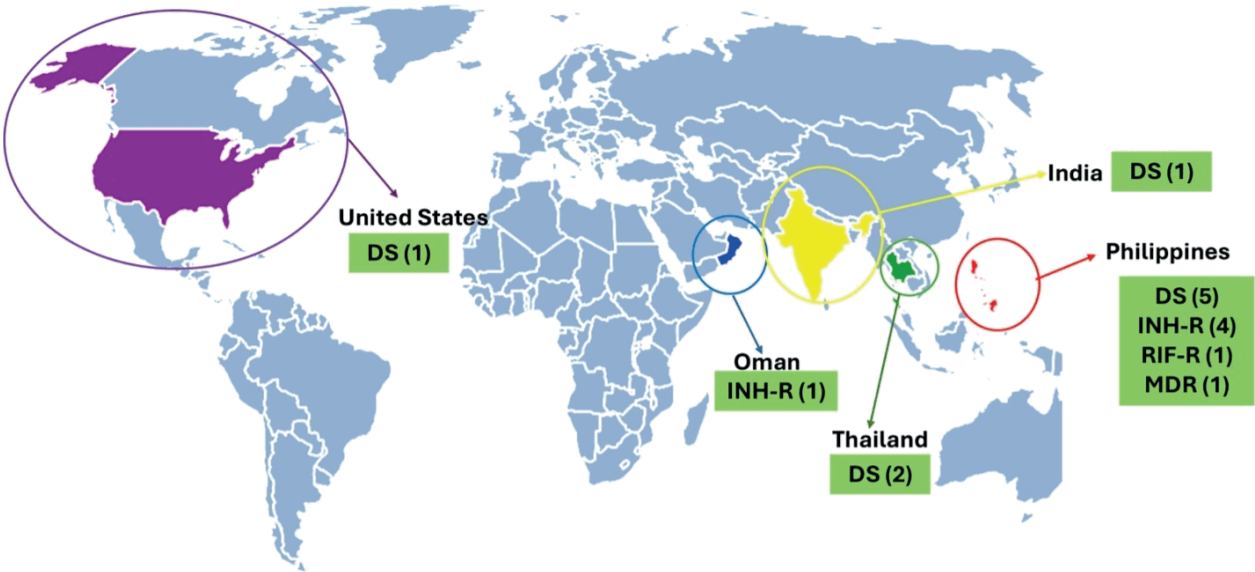


Figure 6. Geographical regions of sixteen samples with a 100% match for the A356T mutation in the INH-R isolate 50886.

Table 4. Locations and drug resistance profiles of *Mtb* strains in the NCBI database with 100% sequence match to *dprE1* of INH-R isolate 50886 (A356T mutation).

Accession	Strain	Location	<i>inhA</i> promoter	<i>inhA</i>	<i>katG</i>	<i>rpoB</i>	Drug resistance profile
CP046308.1	FDAARGOS_751 (Indo-Oceanic T17X)	USA	No	No	R463L	3225 (T→C)	DS
CP029065.1	TBMENG-03	India	No	No	R463L	3225 (T→C)	DS
CP157904.1	H-20-0024	Philippines	G-17T	No	R463L	3225 (T→C)	INH-R
CP157903.1	H-20-0022	Philippines	C-15T	No	R463L	3225 (T→C)	INH-R
CP157905.1	H-21-0019	Philippines	C-15T	No	R463L	3225 (T→C)	INH-R
CP157900.1	H-19-0007	Philippines	No	No	R463L	3225 (T→C)	DS
CP157901.1	H-19-0008	Philippines	No	No	R463L	3225 (T→C)	DS
CP157902.1	H-19-0027	Philippines	No	No	R463L	3225 (T→C)	DS
CP097111.1	MTBCR170020	Thailand	No	No	R463L	3225 (T→C)	DS
CP097110.1	MTBCR170027	Thailand	No	No	R463L	3225 (T→C)	DS
CP130790.1	MTB-Oman-3213318	Oman	C-15T	84 (A→G)	R463L	3225 (T→C)	INH-R
CP040692.1	N0157	Philippines	No	No	R463L	3225 (T→C)	DS
CP069078.1	N0157	Philippines	No	No	R463L	3225 (T→C)	DS
CP009427.1	96121	Philippines	No	S94A	S315T, R463L	3225 (T→C)	INH-R
CP041828.1	3-0090P6C4	Philippines	No	No	R463L	H526S, 3225 (T→C)	RIF-R
CP041827.1	3-0096P6C4	Philippines	C-15T	I21T	R463L	3225 (T→C), S531L	MDR

Note: 3225 (T→C): nucleotide change from T to C in the *rpoB* gene at position 3225, H526S: substitution of serine for histidine of *rpoB* at codon 526, S531L: substitution of leucine for serine of *rpoB* at codon 531, R463L: substitution of leucine for arginine of *katG* at codon 463, S315T: substitution of threonine for serine of *katG* at codon 315, S94A: substitution of alanine for serine of *inhA* at codon 94, I21T: substitution of threonine for isoleucine of *inhA* at codon 21, 84 (A→G): nucleotide changes from A to G in the *inhA* gene at position 84, C-15T: nucleotide change from C to T at *inhA* promoter region (-15), G-17T: nucleotide changes from G to T at *inhA* promoter region (-17), No: no mutation, DS: drug-susceptible strain, INH-R: Isoniazid resistance, RIF-R: Rifampicin resistance, MDR: multi-drug resistance.

For the RIF-R isolates, sequence analysis revealed that two out of six isolates showed no mutations. In comparison, the remaining four isolates carried a mutation at codon 459 (C→T), also found in MDR and INH-R isolates. Interestingly, two of these four RIF-R isolates additionally harbor a G→A mutation at nucleotide position 1198

(G1198A), resulting in an amino acid substitution of isoleucine (I) for valine (V) at codon 400 (V400I) (Figure 7). Notably, no 100% nucleotide identity for this *dprE1* mutant sequence was found in the NCBI database, making this the first uncovering of the V400I amino acid substitution in RIF-R strains.

Sequence alignment of RIF-R isolates

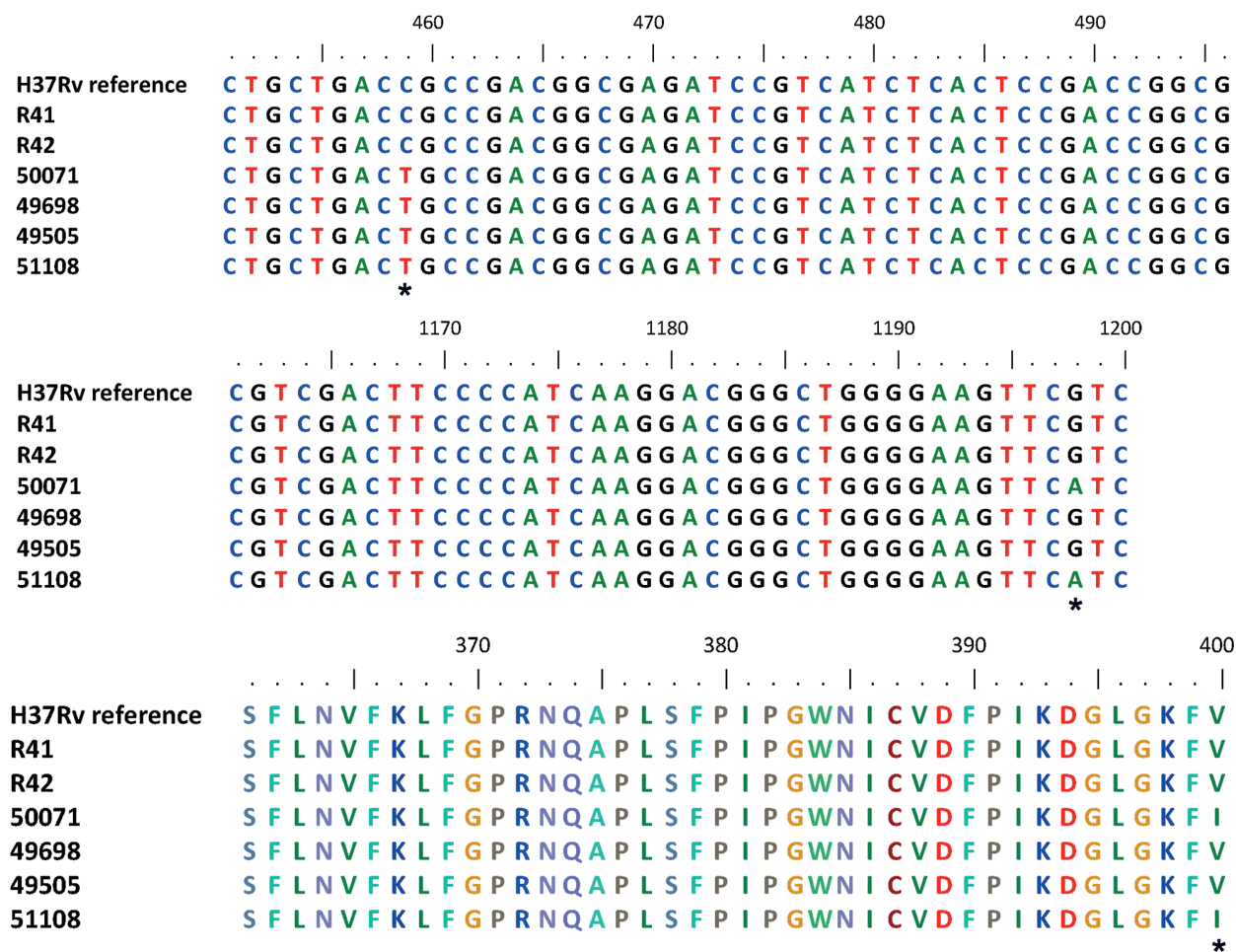


Figure 7. Sequence alignment of *dprE1* in 6 RIF-R clinical isolates against the reference sequence from NCBI - *Mycobacterium tuberculosis* H37Rv, CP003248.2 (H37Rv reference). The position of the nucleotide mutation and amino acid substitution are indicated with *.

The absence of known mutation, the structural impact, and the prevalence of identified mutations

All analyses confirmed that none of the clinical isolates (19/19) had mutations at codon 387 related to BTZ resistance, suggesting all isolates might be susceptible to BTZ compounds. The absence of BTZ resistance-related mutations is particularly significant, as it indicates the continued efficacy of BTZ compounds against these clinical isolates, offering a potential therapeutic option. However, the C→T nucleotide mutation at codon 459 emerged as the most frequently detected mutation, occurring in 14 out of 19 isolates. Consequently, this mutation can be recognized as a common genetic polymorphism across all drug-resistant profiles (73.68%), including 9 MDR strains,

1 INH-R strain, and 4 RIF-R strains, thereby broadening its significance in the context of TB resistance (Figure 8). To study the potential impact of the mutations that were found in INH-R and RIF-R isolates, the amino acid mutation codons were mapped onto the hexagonal crystal structure of the Mtb DprE1 enzyme (PDB ID: 4FEH), obtained from the Protein Data Bank (PDB). Protein structure analysis revealed that both amino acid mutations in INH-R (A356T) and RIF-R (V400I) occur within the substrate/inhibitor-binding domain of the DprE1 enzyme (amino acid residues 197-412) (Figure 9). This suggests the significance of this mutation, which can potentially impact the interaction with the inhibitor binding efficiency.

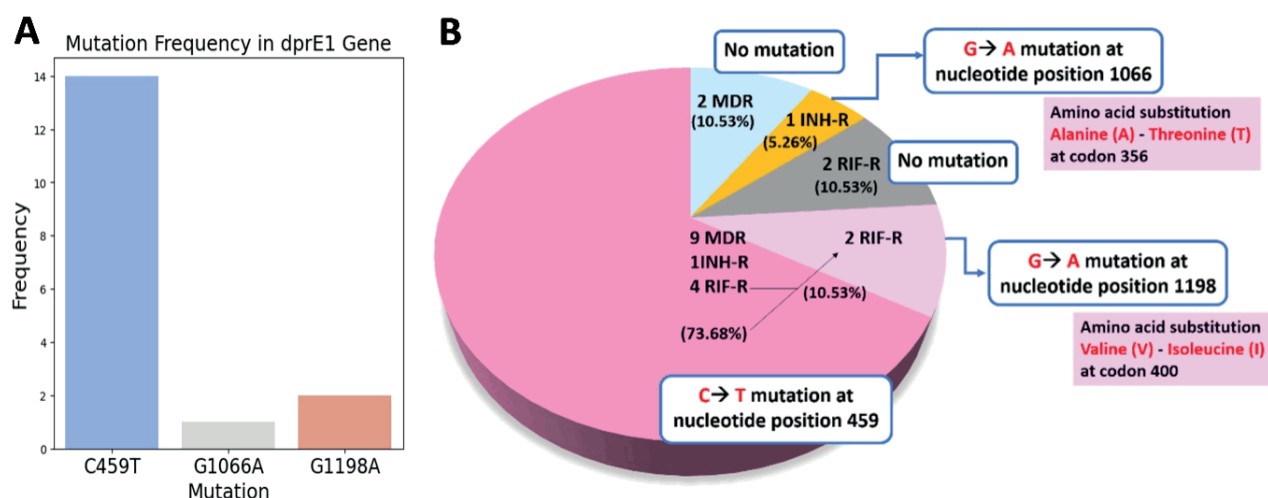


Figure 8. Summary of mutation frequency and distribution of the gene across 19 Mtb clinical isolates. **A:** The bar chart indicated the mutation frequencies of C459T, G1066A, and G1198A nucleotide positions. **B:** The pie chart demonstrated the distribution of mutation patterns among MDR, INH-R and RIF-R clinical isolates, highlighting the nucleotide mutations and their corresponding amino acid substitutions.

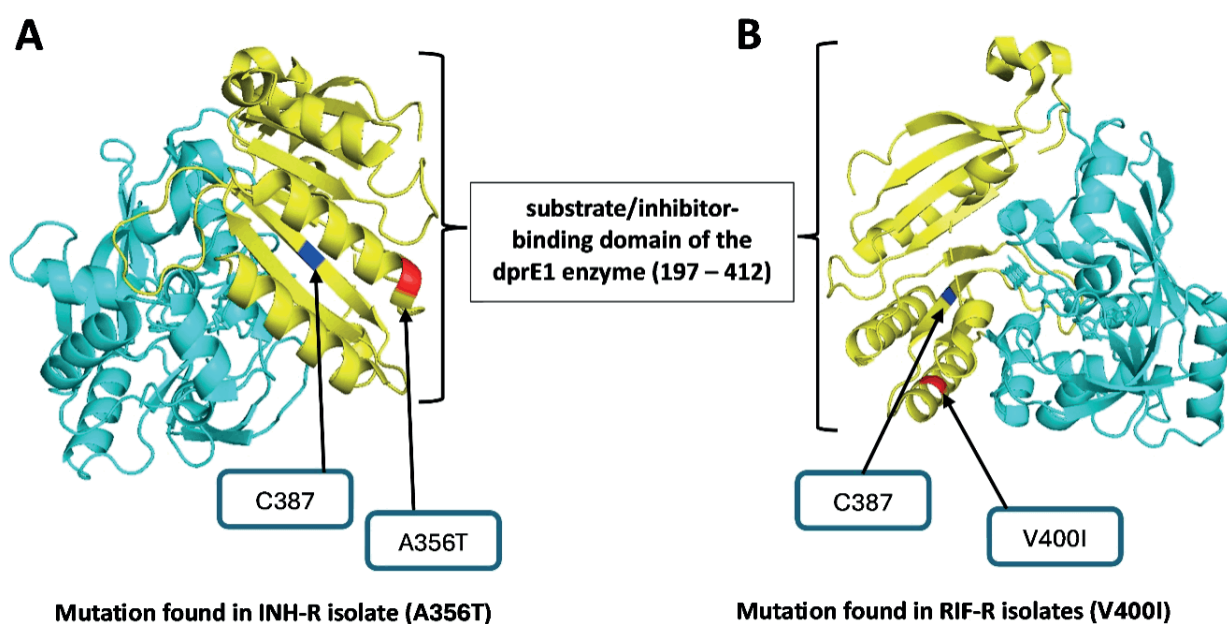


Figure 9. Structural illustrations of amino acid substitutions in the dprE1 enzyme. **(A)** A356T amino acid mutation was identified in an INH-R isolate. **(B)** V400I amino acid mutation was identified in RIF-R isolates. Cysteine 387 residue (C387) was highlighted to indicate a known mutation region associated with resistance to BTZ compounds. The yellow region (amino acid residues 197–412) represents the substrate/inhibitor binding sites of the dprE1 enzyme.

Discussion

In recent years, whole-cell screening has identified several new drug families that target essential proteins involved in cell wall component construction.¹⁸ While cell wall targeting drugs like isoniazid and ethambutol are effective against drug-sensitive Mtb, treating MDR Mtb strains remains a significant challenge, as these strains have developed resistance to the first-line drugs.¹⁹ The discovery of DprE1, due to its essential role of involvement in the fundamental synthesis processes of LAM and AG and

its localization in epimerization processes, identifies it as a valuable cell wall target for MDR-TB. It was demonstrated that silencing of DprE1 caused significant growth arrest, swelling of the bacteria, and cell wall lysis.²⁰ Among the covalent inhibitors of DprE1, the benzothiazinones BTZ043 and PBTZ169 stand out as the most effective compounds, which are currently advancing through phase II clinical trials.²¹ However, mutations in the drug target genes usually confer high resistance to antibiotics.²² A point mutation of a cysteine residue at codon 387 in DprE1 has

been associated with resistance to BTZ compounds in *Mtb*, presenting a challenge to the effectiveness of these promising inhibitors.⁷

Therefore, it is crucial to investigate mutations in the *dprE1* gene, particularly in drug-resistant strains such as MDR-TB, to elucidate potential resistance mechanisms and provide critical information that could enhance the effectiveness of these drugs. However, there needs to be more reports on the sequence analysis and identification of mutations in the *dprE1* gene associated with these promising drugs. In the present study, we focused on the genetic analysis of the *dprE1* gene in various drug-resistant clinical isolates, with particular emphasis on MDR-TB. As expected, no mutations were found at the codon 387 in any of the 19 isolates. This suggests that the present drug-resistant strains remain susceptible to BTZ compounds, which inhibit DprE1 by forming a covalent adduct with this residue. This finding aligns with recent reports from different geographical regions, which showed no mutation of *dprE1* in clinical isolates.¹⁵ Surprisingly, 14 out of 19 isolates show the same nucleotide mutation (C→T) in our work, and this sequence was 100% matched with the sequences from NCBI, mostly in drug-resistant strains. Recent work has also identified this mutation in DS strains, suggesting it might be a genetic polymorphism rather than drug resistance-related.¹⁷ However, investigating its impact on gene expression will be valuable, as silent mutations, although they do not alter the amino acid sequence, can still affect mRNA stability and translation efficiency, potentially influencing treatment response.²³

Noncovalent inhibitors also target the active site of the DprE1 enzyme, but their activity does not rely on the presence of cysteine.²⁴ Beyond covalent bond formation, studies have shown that the DprE1 enzyme can also be inhibited through hydrophobic interactions, such as at residue 275 by the binding of selamectin.^{25,26} TCA1 is a noncovalent inhibitor that does not possess the nitro group required for covalent binding to codon 387. Interestingly, a DprE1 mutation with a Y314C substitution, which confers resistance to TCA1, has been found to remain sensitive to BTZ. This suggests that TCA1 likely interacts with DprE1 through a mechanism different from BTZ.^{10,18} Moreover, earlier work demonstrated that mutation of the 16S rRNA gene at positions 1401, 1402, and 1484 is associated with cross-resistance between Amikacin, Kanamycin, and Capreomycin in *Mtb*.²⁷ These all highlight that other residues within DprE1 are also crucial for interactions with inhibitors, and mutation of these residues might potentially develop drug resistance.

Our study identified nucleotide mutations that led to amino acid substitutions in one INH-R (A356T) and two RIF-R (V400I) isolates. Although these mutations do not correspond to previously identified resistance sites for either covalent or noncovalent inhibitors, they are located within the substrate/inhibitor binding domain of DprE1. This domain is essential for the enzyme's function, and mutations within it could alter the enzyme's interaction with substrates or inhibitors, potentially contributing to the development of drug resistance. When the mutation

sequences were analyzed using the NCBI database, it was found that sixteen *Mtb* strains from different regions showed a 100% identity with the *dprE1* mutant sequences in an INH-resistant isolate. These specific mutations are common and have been identified in various geographical locations, suggesting widespread occurrence potentially driven by clonal expansion through migration or selective antibiotic pressure in regions with high drug use. In addition, this mutation was also observed in DS strains, and it could pose a threat to treatment if it is related to drug resistance.

In contrast, no 100% matches were found for the sequences from the RIF-resistant isolates. This absence of exact matches highlights that the mutations present in the RIF-resistant strains might represent novel or less well-characterized genetic variations. Therefore, deeper investigation into these mutations is necessary to determine how they might affect the overall resistance profile of the isolates.

An interesting finding is that amino acid substitutions in DprE1 were predominantly observed in both INH-resistant (INH-R) and RIF-resistant (RIF-R) isolates. Since *Mtb* does not acquire resistance through horizontal gene transfer, the evolution of multi-drug resistance is influenced by pre-existing drug resistance.²⁸ Previous reports indicate that strains with an isoniazid mono-resistant background have a 15-fold higher risk of developing rifampicin resistance than wild-type strains. This suggests that DprE1 mutations may arise from subsequent mutations in mono-resistant strains.²⁹ Further research should focus on mono-resistant isolates to elucidate the mechanisms driving these additional mutations.

Limitation

This study was conducted on a limited number of clinical isolates, and larger sample sizes are needed for a comprehensive understanding of the mutation patterns in the *dprE1* gene. Furthermore, the isolates investigated in this study were collected exclusively from northern Thailand, which may not fully represent the different mutations that might be prevalent in other regions. Functional studies of the amino acid changes in these strains will be necessary to understand their susceptibility to the proposed drugs.

Conclusion

This research revealed that Cys387 mutation was not observed in the drug-resistant isolates, supporting the positive impact of BTZ compounds in eradicating MDR. While a common synonymous substitution was identified in several isolates, its presence in drug-sensitive strains suggests a potential genetic polymorphism. However, detecting non-synonymous substitution in different residues of INH-R and RIF-R isolates indicates that current circulating mono resistance strains can acquire additional mutations in *dprE1* genes. These amino acid changes in the inhibitor binding domain could alter drug interaction, underscoring the need for further investigation to determine their impact on treatment efficacy.

Conflicts of interest

The authors declare no conflict of interest.

Funding

This work was funded by the National Research Council of Thailand (NRCT) [NRCT5- RSA63004-14], the CMU research fellowship program, and the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program (RGJ PhD) [PHD/0114/2561].

Acknowledgements

The first author would like to thank the Presidential Scholarship, Chiang Mai University, for supporting the master's degree program in Medical Technology and the Faculty of Associated Medical Sciences, Chiang Mai University, for providing facilities and equipment.

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