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Molecular characterization of the Pyrazinamidase (PZase) drug target protein in multidrug-resistant mycobacterium tuberculosis clinical isolates

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ABSTRACT: Pyrazinamide (PZA) is a first-line anti-tuberculosis (TB) drug whose activity depends on the pyrazinamidase (PZase) enzyme encoded by the pncA gene in Mycobacterium tuberculosis. Mutations in pncA that impair PZase function are strongly associated with PZA resistance. In this study, 20 multidrug-resistant TB (MDR-TB) clinical isolates were analyzed to assess the molecular diversity of the PZA target protein. The pncA gene was successfully amplified by PCR (0.6 kb) and sequenced using the Sanger method. Mutations in pncA were detected in 85% of the isolates, consisting of substitutions, insertions, deletions, and stop-codon alterations. Many of the mutations led to amino acid changes or frameshift events in the PZase protein. Seven MDR-TB isolates carried substitution mutations that altered the PZase amino acid sequence, while three isolates harbored insertion mutations and three had deletion mutations, leading to

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frameshifts in the protein. In addition, four isolates exhibited stop-codon mutations that were converted into nonstop codons. Five substitution mutations (C175T, G289A, C83A, T173G, and G356T) were identified as novel and resulted in the amino acid changes Leu59Phe, Gly97Ser, Ala28Asp, Phe58Cys, and Trp119Leu, respectively. These findings underscore the strong association between pncA mutations and PZA resistance and highlight the need for further studies to elucidate the structural and functional impacts of these mutations on PZase.

1. INTRODUCTION

Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis* (MTB) (Ronan et al., 2022). The bacterium is rod-shaped, acid-fast, and characterized by a complex cell wall composed of arabinogalactan surrounded by lipid layers containing mycolic acids and sulfolipids (Cole et al., 1998; Jankute et al., 2015). The high lipid content contributes to its resistance to staining and antibiotics, as well as its ability to survive under harsh conditions such as acidity, alkalinity, low oxygen levels, and within macrophages (Tobin & Tristram, 2024). Although TB primarily affects the lungs, it may also spread to other organs such as the pleura, lymph nodes, and bones (Ministry of Health, Republic of Indonesia, 2023).

According to the World Health Organization (WHO, 2024a), more than 10.8 million new TB cases and 1.25 million deaths were reported globally in 2023, including 161,000 deaths among people with HIV co-infection. Over 95% of cases occur in developing countries, with Indonesia ranking second after India, accounting for approximately 10% of the global burden. The Ministry of Health, Republic of Indonesia (2023) reported 724,000 notified cases, of which 83% were pulmonary TB. The prevalence is higher among males (57.5%) than females (42.5%), likely due to greater exposure to risk factors such as smoking, air pollution, and lower treatment adherence (Ministry of Health, Republic of Indonesia, 2023). Despite the decline in mortality following the COVID-19 pandemic, TB remains the leading cause of death from a single infectious agent—almost twice that of HIV/AIDS (WHO, 2024a). Globally, progress toward the WHO *End TB Strategy* targets remains insufficient, with only an 8.3% reduction in new cases and a 23% decline in deaths since 2015, far below the 2025 targets of 50% and 75%, respectively (WHO, 2024a).

TB transmission occurs through droplet nuclei (1–5 μm) expelled when an infected person coughs, sneezes, or speaks. Inhaled droplets that reach the alveoli initiate infection as MTB interacts with alveolar macrophages (Khawbung et al., 2021; Xia et al., 2015). While most infections are contained by host immunity, some bacilli remain dormant, leading to latent TB infection (LTBI), which may reactivate under immunocompromised conditions such as HIV co-infection, diabetes, cancer, or immunosuppressive therapy (Khawbung et al., 2021).

The emergence of drug-resistant tuberculosis (DR-TB) poses a major challenge to global TB control. WHO (2024a) classifies resistance into isoniazid-resistant TB (Hr-TB), rifampicin-resistant TB (RR-TB), multidrug-resistant TB (MDR-TB), pre-extensively drug-resistant TB (Pre-XDR-TB), and extensively drug-resistant TB (XDR-TB). Resistance arises primarily from genetic mutations in drug-target genes, altering protein structure and reducing drug efficacy, or from external factors such as poor treatment adherence, irrational drug use, delayed diagnosis, and limited healthcare access. Prolonged treatment duration also contributes to resistance, as standard regimens typically last 4–6 months for drug-sensitive TB and ≥18 months for resistant cases (WHO, 2024a).

This antibiotic regimen plays a crucial role in the success of TB control, with treatment success rates of approximately 85–95% in drug-sensitive TB cases (WHO, 2024a). One of the first-line drugs in the TB treatment regimen is pyrazinamide (PZA), which is essential for shortening the duration of therapy and eliminating bacteria during the latent phase. This makes PZA a potent and critical component of both drug-sensitive and DR-TB treatment regimens.

PZA is a prodrug that must be converted into its active form by the enzyme pyrazinamidase (PZase) to make a therapeutic effect in mycobacteria. The enzyme catalyzes the conversion of PZA into pyrazinoic acid (POA). Resistance to PZA is largely attributed to mutations in the *pncA* gene, which encodes the PZase enzyme (Purkan et al., 2024; Ramirez-Busby and Valafar, 2015; Scorpio et al., 1997). Mutations in the *pncA* gene lead to the loss of PZase enzymatic activity, preventing the conversion of PZA into its active form and thereby reducing the drug's effectiveness, ultimately resulting in PZA resistance in MTB.

Based on geographical studies, variations in the *pncA* gene mutations among clinical isolates of MTB show significant diversity across different regions. This indicates that patterns

of resistance and genetic mutations may vary between areas, highlighting the importance of conducting studies on specific mutations in Indonesia. In relation to these distinctive characteristics, analyzing the mutation profile of the *pncA* gene in local clinical isolates of PZA-resistant MTB is essential to establish region-specific genetic markers.

Numerous clinical isolates of MTB have demonstrated a phenotype resistant to PZA; nevertheless, the fundamental genotypic mechanisms driving this resistance are yet to be elucidated. Profiling of the *pncA* gene, which encodes the target enzyme of PZA, in these local clinical isolates is essential to establish mutation data specific to local strains. Such data can support the development of more effective treatment strategies and enhance TB control efforts at both regional and national levels. Furthermore, it is important to identify genetic markers that can facilitate molecular diagnosis and guide more precise therapeutic strategies against PZA resistance. The *pncA* gene profiling from PZA-resistant clinical isolates will be conducted using PCR amplification and sequencing methods, employing specifically designed primers to amplify the *pncA* coding region.

2. MATERIALS AND METHODS

2.1. Samples

The samples used in this study were genomic DNA extracts from MDR-TB isolates. The samples were obtained from the Biomedical Research Center Laboratory, National Research and Innovation Agency (BRIN), Cibinong, Bogor, West Java. The genomic DNA was derived from clinical isolates of *M. tuberculosis* that had undergone TB drug susceptibility testing (DST).

2.2. Chemicals

The materials used in this study were Tris base, glacial acetic acid, ethylenediaminetetraacetic acid (EDTA), distilled water, NaOH, HCl, ddH2O, agarose (1st BASE Singapore), primers F*pncA*Nde and R*pncA*Bgl (Genetika Science) (Table 1), MyTaq HS Red Mix (Meridian Bioscience, UK), and ethidium bromide (EtBr).

Table 1
Primers used in the PCR and sequencing.

No	Primer's Name	Nucleotide of Primers
1	*FpncA*NdeI	5'-GAGCATATGCGGGCGTTGATCATC-3'
2	*RpncA*Bgl	5'-GAAGATCTGGAGCTGCAAACCAACTC-3'

2.3. Equipment

The equipment used in this study included a pH meter (Mettler Teledo), an analytical balance (Ohaus-Adventurer), a hotplate and magnetic stirrer (Thermo Scientific), an autoclave (GEA®Medical YX-21LM), an oven (Memmert), a D1524R High-Speed Refrigerated Centrifuge, a PCR apparatus (Biometra Tadvanced), an agarose gel electrophoresis kit, a UV transilluminator (Herolab UVT-20 M/W), a gel documentation system (Syngene InGenius), 1.5 mL microtubes, 0.5–10 µL, 10–100 µL, and 100–1000 µL micropipettes, PCR tubes, a freezer, a Sharp refrigerator, and other glassware commonly used in chemistry laboratories.

2.4. Amplification of pncA gene

The *pncA* gene fragment was amplified using a polymerase chain reaction (PCR) kit with the primer pair F*pncA*Nde and R*pncA*Bgl in composition in Table 2. An illustration of primer attachment to the *pncA* gene nucleotide sequence is shown in Figure 1. Before the PCR stage, the primers were diluted to create a 100 µM primer stock solution with ddH_2O, following the purchase protocol. Then, a 20 µM primer working solution was prepared for PCR by diluting the previously prepared primer stock solution.

The pncA gene amplification process was carried out using a PCR apparatus for 30 cycles under the process conditions listed in Figure 2. The PCR process began with a pre-denaturation reaction at 95°C for 5 minutes, followed by 30 cycles of gene amplification. Each cycle consisted of three steps: denaturation for 1 minute, annealing at 53°C for 30 seconds, and polymerization at 72°C for 45 seconds. The final PCR step was completed with a post-extension step at 72°C for 5 minutes, followed by a temperature drop of 16°C. The amplification results were analyzed by 1.5% (w/v) agarose gel electrophoresis.

Table 2
PCR reaction mixture composition.

No	Component	Vol (µL)
1	MyTaq HS Red Mix	25
2	DNA template	1
3	F*pncA*NdeI Primer (20 uM)	1
4	R*pncA*Bgl Primer (20 uM)	1
6	ddH_2O	22
	Total of volume	50

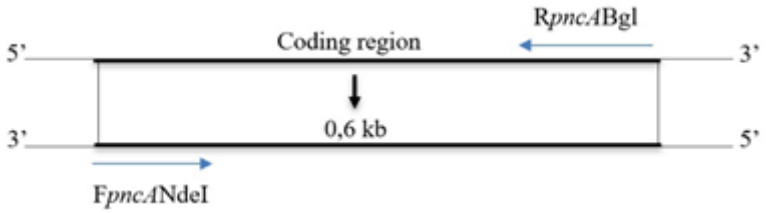

Figure 1. Schematic of primer annealing to the coding region of the *pncA* gene.

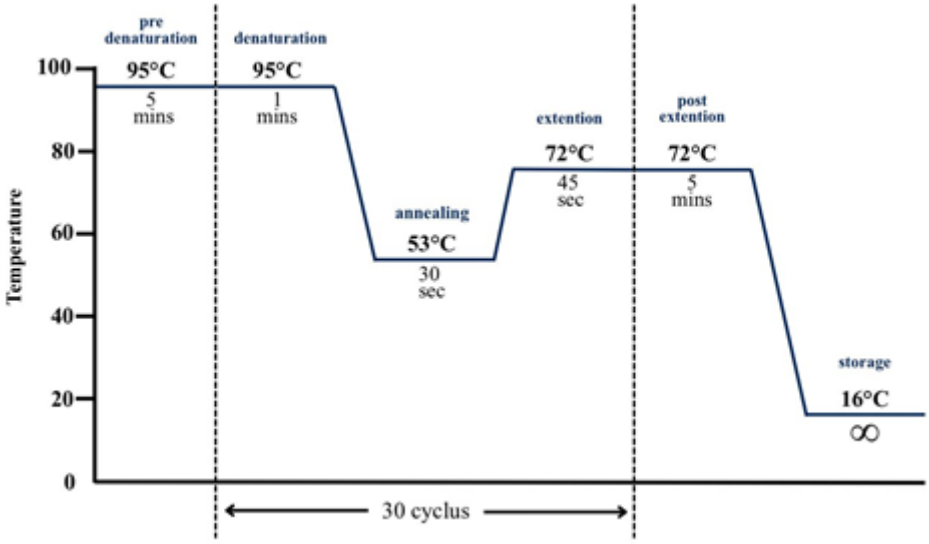

Figure 2. PCR process and reaction steps.

2.5. Detection of DNA fragments with agarose gel electrophoresis

This step begins with the preparation of a 50X TAE buffer stock solution (100 mL) by mixing 24.2 g of Tris base, 5.71 mL of glacial acetic acid, and 10 mL of EDTA (0.5 M) pH 8. First, the EDTA solution was prepared by weighing 1.8612 g of EDTA and dissolving it in distilled water to 10 mL. The EDTA solution was then mixed with the other components in a beaker and homogenized using a magnetic stirrer. The pH of the solution was checked using a pH meter by adding NaOH or HCl until it reached pH 8. The solution was then transferred to a 100 mL volumetric flask, and distilled water was added to the mark. The 50× TAE buffer solution was sterilized using an autoclave and stored in a dark bottle. The buffer solution used for electrophoresis is 1× TAE buffer, made by diluting a previously prepared 50× TAE buffer stock solution.

The electrophoresis stage consists of two steps: agarose gel preparation and DNA separation. A 1.5% agarose gel is prepared by dissolving 0.9 g of agarose in 60 mL of 1X TAE buffer. The mixture is placed in an Erlenmeyer flask covered with aluminum foil. The agarose solution is heated to boiling using a hotplate while stirring (magnetic stirrer) until dissolved. Once warm, 3 µL of EtBr is added to the agarose solution and homogenized. The solution is poured into an agarose gel mold fitted with a comb and left to solidify. Once solidified, the comb is removed and the agarose gel is transferred to an electrophoresis vessel filled with 1× TAE buffer until the gel is submerged. The electrophoresis process begins by adding 15 µL of DNA marker into the first gel well using a micropipette. Next, 15 µL of the sample was added to the next gel well. Electrophoresis was run for 60 minutes at 80 volts using a Bio-Rad mini-sub DNA electrophoresis cell. After the run was complete, the agarose gel was removed from the electrophoresis chamber and the resulting bands were observed using a UV transilluminator at a wavelength of 205 nm. The electrophoresis results were documented using a digital camera.

2.6. Nucleotide sequencing and mutation analysis

The nucleotide sequence of the *pncA* gene from the PCR was determined using the dideoxy-Sanger method using several appropriate primers. Nucleotide sequence determination was performed using the analysis services of 1st BASE, Singapore. Mutations in the *pncA* gene of the *M. tuberculosis* isolate were identified using the DNASTAR program by comparing the nucleotide sequences with the *pncA* gene of the *M. tuberculosis* H37Rv (wild-type) and the *pncA* gene of the *M. tuberculosis* (MDR) isolate through alignment. The identified data were then translated in silico to determine any changes at the protein level. Gene sequencing was performed using the primers F*pncA*Nde and R*pncA*Bgl.

2.7. Nucleotide alignment

Nucleotide sequence alignment was performed between the sequenced *pncA* gene fragment and the reference *pncA* gene from GenBank using the DNASTAR program via the SeqMan Sequences feature. The initial step was to input the sequenced and reference nucleotide sequences into the EditSeq feature as a database. Then, multiple alignment was performed using the SeqMan feature to identify nucleotide differences between the sequenced and reference sequences that indicate mutations.

3. RESULTS

This study was designed to detect *pncA* gene diversity in several clinical isolates of *M. tuberculosis* MDR-TB. Twenty clinical isolates were tested for drug resistance, and the results are shown in Table 3. Of the 20 sputum samples from TB patients, 15 isolates appear to be positive for BTA tests. The BTA test does not show a high accuracy, because sometimes samples that show BTA are negative but when bacterial culture is carried out, it shows the presence of *M. tuberculosis* cultures growing. In Table 3, there are five samples having

Table 3
Profile of clinical isolate samples of *M. tuberculosis* MDR-TB.

No	BTA Check	Culture	Direct Susceptibility Test (DST)							
			S 1.0 (µg/mL)	I 0.1 (µg/mL)	R 1.0 (µg/mL)	E 5.0 (µg/mL)	P 40 (ug/mL))	O 2.0 (µg/mL)	A 1.0 (µg/mL)	K 5.0 (µg/mL)
1	8 AFB	positive	R	R	R	R	R	S	S	R
2	1+	positive	S	R	R	S	R	R	S	S
3	2+	positive	S	R	R	S	R	R	S	S
4	1+	positive	R	R	R	R	R	R	R	R
5	5 AFB	positive	R	R	R	S	R	S	R	S
6	1+	positive	R	R	R	S	R	S	S	S
7	Negative	positive	R	R	R	S	R	R	S	S
8	8 AFB	positive	R	R	R	S	R	R	S	S
9	1+	positive	R	R	R	R	R	R	R	R
10	Negative	positive	R	R	R	R	R	R	R	R
11	2+	positive	R	R	R	S	R	R	S	S
12	Negative	positive	R	R	R	R	R	R	R	R
13	1+	positive	S	R	R	S	R	S	S	S
14	1+	positive	S	R	R	S	R	R	S	S
15	Negative	positive	R	R	R	S	R	R	S	S
16	Negative	positive	R	R	R	S	R	S	S	S
17	1+	positive	R	R	R	S	R	R	S	S
18	1+	positive	R	R	R	R	R	R	R	R
19	3+	positive	R	R	R	S	R	R	R	S
20	3+	positive	R	R	R	R	R	R	R	R

Note: O = Ofloxacin; A = Amikacin; K = Kanamycin; S = Streptomycin; I = Isoniazid; R = Rifampin; E = Ethambutol; P = pyrazinamide.

negative BTA check, but each sample showed a positive result for *M. tuberculosis* culture.

Several isolates demonstrated diverse resistance profiles to multiple anti-TB drugs (Table 3). Notably, all isolates were resistant to first-line drugs, namely, rifampin, isoniazid, and PZA. This resistance phenotype needs to be confirmed by molecular analysis. In this case, the molecular analysis focused on the *pncA* gene, the target gene for PZA. This drug is reported to be able to kill both dormant and semi-dormant *M. tuberculosis* bacilli. Another reason is that this drug is always given to patients alongside isoniazid and rifampin, always in the first 2 months of treatment.

3.1. Molecular detection of the pncA gene in the clinical isolate of *M. tuberculosis* MDR-TB by PCR

The *pncA* gene, which encodes the PZase enzyme in clinical isolates of MDR-TB *M. tuberculosis*, was detected by PCR, and the resulting fragments were then identified for nucleotide sequence using sequencing techniques. The PCR process, carried out using forward and reverse pairs, was designed to amplify the 0.6 kb coding region of the *pncA* gene.

PCR amplification results, after being examined by agarose gel electrophoresis, showed the presence of the targeted band at 0.6 kb (Figure 3 and 4). This result indicated that the *pncA* gene from these clinical isolates was successfully amplified. The nucleotide sequence of the DNA fragments was then determined through a Sanger sequencing reaction using the same two primers used in the PCR process.

3.2. Molecular diversity of drug target proteins (PZase) in clinical isolates

To assess the molecular diversity of the *pncA* gene encoding PZase, sequencing was performed using two primers, F and R. The sequencing process was performed using the Sanger dilution method. DNA fragments with nucleotide sequences (Table 3) have been sent to 1stBase, but the sequencing data are not yet complete. Once the nucleotide sequence of the PZase gene is complete, mutation profile analysis will be performed to determine which mutants are novel, meaning those not yet reported in published articles. This mutation profile for PZase is crucial for the genetic design of markers in this research.

For clinical isolate 4 that resistant to PZA showed a mutation in *pncA* gene, that is, G289A, then change its protein PZase for Gly97Ser (Figure 5). Meanwhile, isolate 13, which was also resistant to PZA, carried a C83A mutation at the gene level, leading to an amino acid change from alanine to aspartic acid at position 28 (Ala28Asp) in the PZase protein (Figure 6). Details of mutation for 20 isolates and the PZA resistance characteristics can be seen in Table 4.

Of the 20 MDR-TB isolates resistant to PZA, 17 harbored mutations in the *pncA* gene, whereas 3 isolates did not. Notably, isolates 1, 6, and 16 showed no detectable mutations in *pncA* despite exhibiting phenotypic resistance to PZA. In MTB, resistance to PZA is predominantly caused by mutations in the *pncA* gene. However, in a limited number of cases, PZA resistance may also be associated with mutations in other genes, such as ribosomal protein S1 (*rpsA*) and aspartate decarboxylase (*panD*). Therefore, the

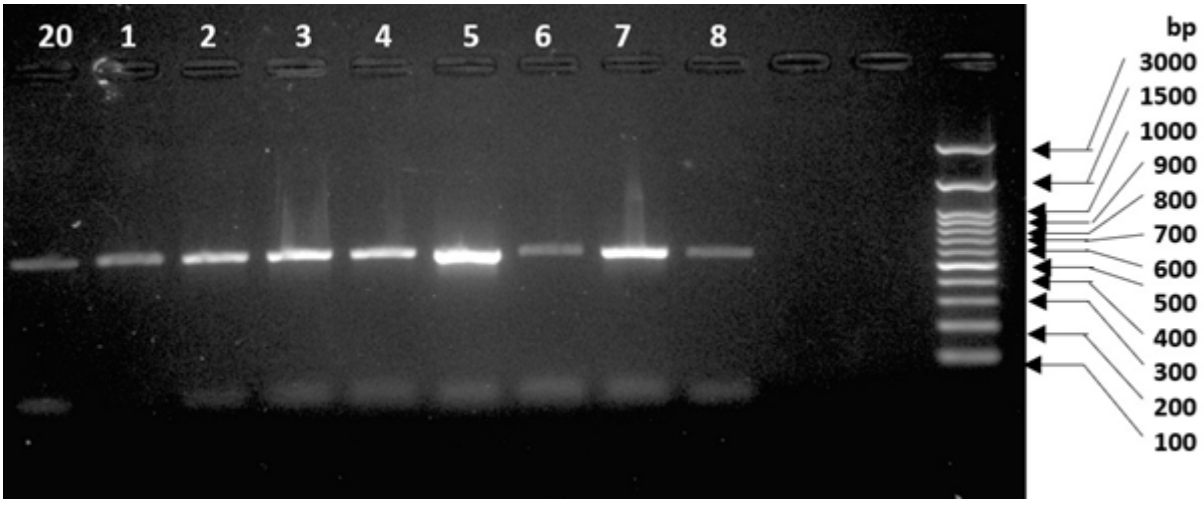

Figure 3. Electroforegram of fragment DNA for *pncA* gene from isolate 1–8 and 20. The band of *pncA* appears at 0.6 kb.

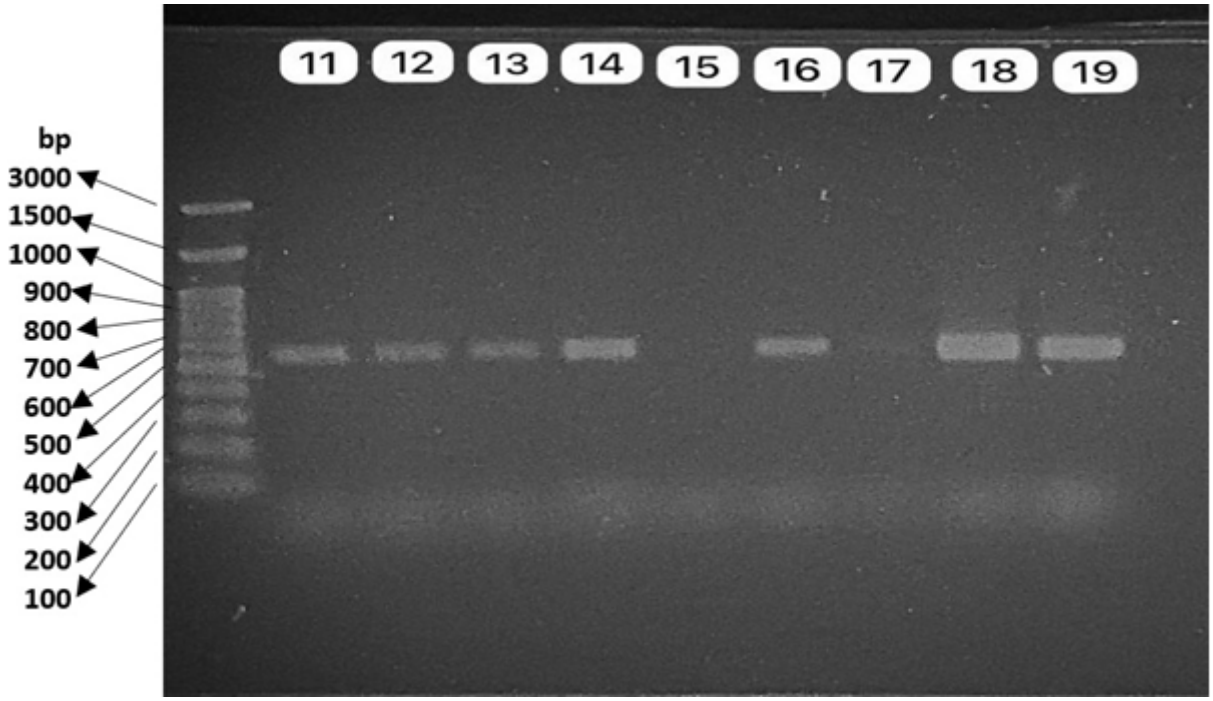

Figure 4. Electroforegram of fragment DNA for *pncA* gene from isolate 11–19. The band of *pncA* appears at 0.6 kb.

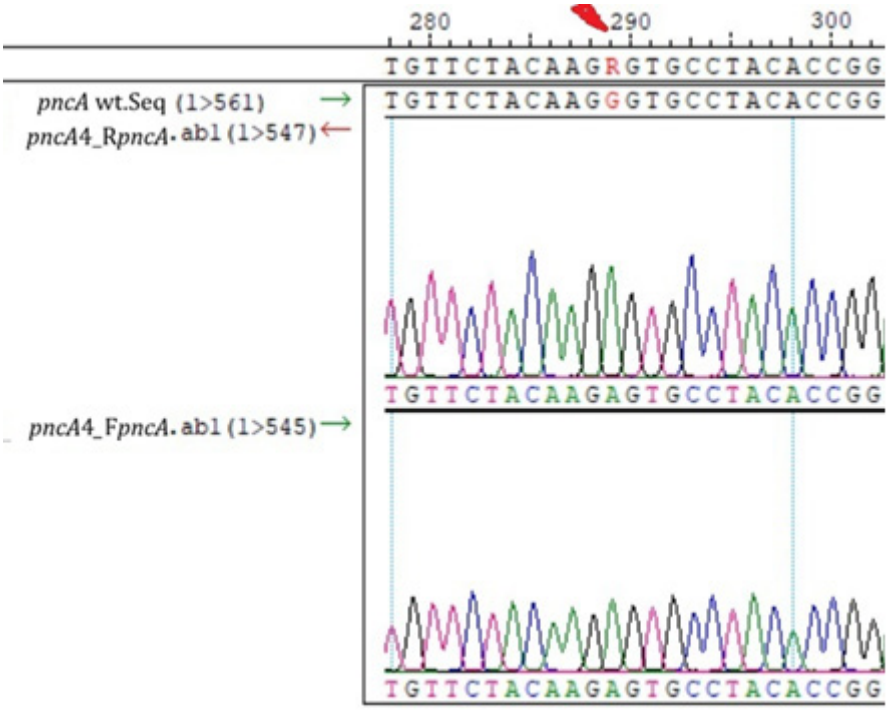

Figure 5. Electrophoregram of mutation analysis on *pncA* from isolate 4. The analysis is compared with *pncA* genbank that is represented as PZA sensitive. The mutation takes place at G289A for the *pncA* gene, then changes the amino acid Gly97Ser in the protein level of PZase.

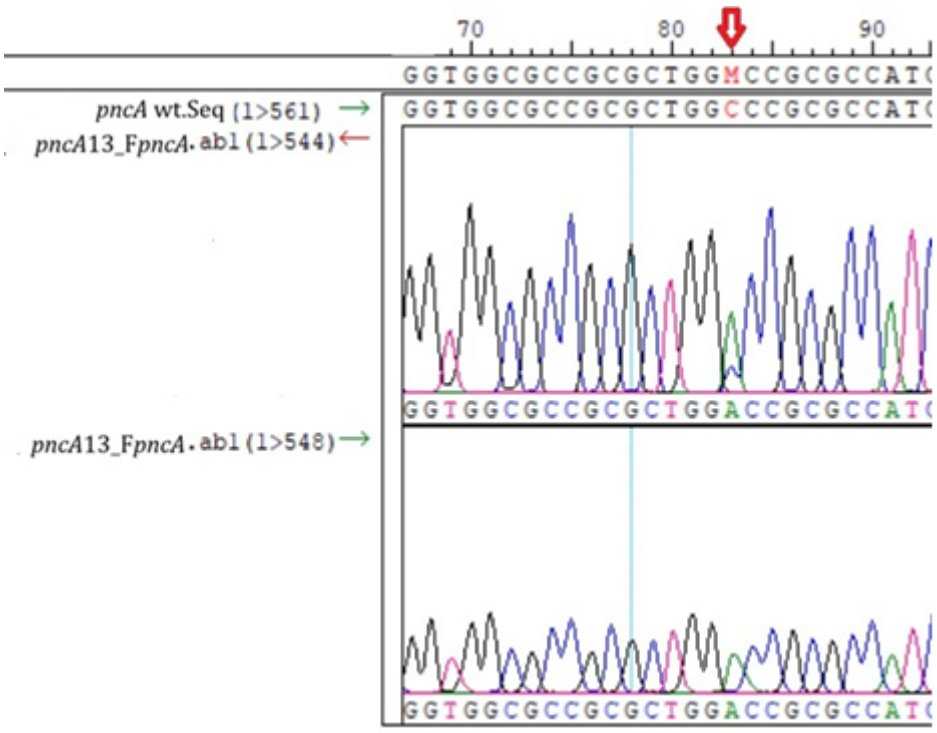

Figure 6. Electrophoregram of mutation analysis on *pncA* from isolate 13. The analysis is compared with *pncA* genbank that is represented as PZA sensitive The mutation takes place at C83A for *pncA* gene, then changes the amino acid Ala28Asp in the protein level of PZase.

Tablel 4

Molecular profile of the *pncA* gene in MDR-TB isolates from PCR products.

Isolate_Sensitivity to PZA (40 µg/mL)	*pncA*	Σ Fragment DNA	Mutations	*pncA* Gene	Protein of PZase
1	R	√	1	ND	ND
2	R	√	1	C175T (CTC→TTC) G432A (GAG→GAA)	Leu59Phe Glu144Glu
3	R	√	1	Insertion T at position 3	Misframing protein
4	R	√	1	G289A (GGT→AGT)	Gly97Ser
5	R	√	1	Deletion G 520	Misframing protein
6	R	√	1	ND	ND
7	R	√	1	Deletion G at position 5	Misframing protein
8	R	√	1	G5T (CGG →CTG)	Arg2Leu
9	R	√	1	T559G	Stop 186 Glu
10	R	√	1	T559A (TGA→AGA)	Stop 186 Arg
11	R	√	1	C4G (CGG→GGG)	(Arg2Gly)
12	R	√	1	Deletion G517	Misframing protein
13	R	√	1	C83A (GCC→GAC)	Ala28Asp
14	R	√	1	T173G	Phe58Cys
15	R	√	1	T559A	Stop 186 Arg
16	R	√	1	ND	ND
17	R	√	1	Insertion A at position 84	Misframing protein
18	R	√	1	G356T	Trp119Leu
19	R	√	1	Insertion A173	Misframing protein
20	R	√	1	T559A (TGA→AGA)	Stop186Arg

Table 5
Summary statistics of mutation types.

Mutation category	Number of isolates	Percentage (%)	Previously reported	Novel mutations	Common mechanism
Substitutions	7	35%	2 (Leu85Pro, Asp12Ala)	5 (Leu59Phe, Gly97Ser, Ala28Asp, Phe58Cys, and Trp119Leu)	Spontaneous base substitution
Insertions	3	15%	Similar positions reported	Novel specific positions	Slipped-strand mispairing
Deletions	3	15%	Similar deletions documented	Novel specific positions	Replication slippage; illegitimate recombination
Stop codon mutations	4	20%	Rare but documented type	Novel specific changes	Point mutations under drug selection
Wild-type	3	15%	N/A	N/A	No mutation detected
Total with mutations	17	85%	Multiple	Multiple	Various mechanisms

PZA resistance observed in isolates 1, 6, and 16 could be attributed to mutations in these alternative genes, although further molecular investigation is required to confirm this possibility.

Observations in the initial study focused solely on the *pncA* gene, the primary target associated with PZA resistance. Among the 17 MDR-TB isolates resistant to PZA, mutations in the *pncA* gene varied in type, including substitutions, insertions, and deletions. Seven MDR-TB isolates had base substitution mutations in the *pncA* gene, altering the amino acid sequence of their PZase protein (isolates 2, 4, 8, 11, 13, 14, and 18). Three isolates had N-base insertions in the *pncA* gene (isolates 3, 17, and 19), leading to misframing of the PZase protein. There are three isolates carrying deletion mutations, namely, at isolates of 5, 7 and 12. All deletion mutations disrupt the reading frame, preventing proper protein formation. Furthermore, there are 4 isolates who stop codon mutations to functional amino acids, namely, isolates 9, 10, 15, and 20 (Table 4). Mutations at the stop codon prevent proper protein termination, resulting in nonfunctional enzymes. All of these *pncA* gene mutations are closely involved in the emergence of drug resistance properties in the studied isolates. Unlike other drug resistance genes, *pncA* shows no single dominant mutation hotspot, suggesting multiple independent genetic pathways to PZA resistance. Comparative mutations from the observed isolates, their frequency, and impact are listed in Table 5.

Five new substitution mutations (29% of all substitution mutations) consist of C175T, G289A, G83A, T173G, and G356T that change the amino acids of Leu59Phe, Gly97Ser, Ala28Asp, Phe58Cys, and Trp119Leu, respectively, and are classified as novel mutations (Table 5). The type mutations have existed in published data, so they expand the global *pncA* mutation database.

The comparative analysis between the mutations identified in this study and previously reported *pncA* gene mutants from various sources is presented in Table 6. Furthermore, the table includes additional information regarding events that arise as consequences of the mutational effects.

4. DISCUSSION

The emergence of MDR-TB poses a significant threat to global public health, necessitating a comprehensive understanding of the molecular mechanisms underlying drug resistance. This study investigated the molecular characterization of the PZase drug target protein in MDR MTB clinical isolates, with particular focus on mutations in the *pncA* gene that encodes this critical enzyme. Our findings provide important insights into the genetic diversity and mutation patterns associated with PZA resistance in MDR-TB cases.

4.1. Prevalence of pncA gene mutations in MDR-TB Isolates

Although based on a limited number of isolates, the 85% mutation rate observed in this study (Table 5) falls within the range reported in previous studies, where pncA mutations have been detected in approximately 72–97% of PZA-resistant strains. This observed prevalence is comparable to previous reports that have implicated pncA mutations as an important contributor to PZA resistance

Table 6
Comparative analysis of mutations identified in the present study with previously reported mutations.

No	Mutation in		Novel/reported	Year first reported	Clinical/functional significance	Proposed mechanism	Refs
	pncA	PZase					
1	C175T	Leu59Phe	Novel	Present study (2025)	Likely disrupts catalytic domain; reduced enzyme activity	Spontaneous base substitution during DNA replication	This study; (1,2)
2	G289A	Gly97Ser	Novel	Present study (2025)	Alters protein flexibility; may affect active-site geometry	G→A transition due to replication error	This study; (4,5)
3	G83A	Ala28Asp	Novel	Present study (2025)	Introduces charged residue; affects protein stability	DNA polymerase error	This study; (7,8)
4	T173G	Phe58Cys	Novel	Present study	Disrupts hydrophobic core; affects folding	Transversion mutation	This study; (10,11)
5	G356T	Trp119Leu	Novel	Present study (2025)	Loss of conserved aromatic residue; structural destabilization	Replication error	This study; (13,14)
6	CTG→CCG	Leu85Pro	Reported	2000	High-frequency resistance mutation; complete loss of activity	Spontaneous point mutation	(16–18)
7	GAT→GCT	Asp12Ala	Reported	1997	Disrupts metal binding essential for catalysis	Base substitution	(7,8,19)
8	Insertion A at position 127	Frameshift	Similar reported	2010	Truncated, nonfunctional protein	Slipped-strand mispairing	(21–23)
9	Deletion T at position 383	Frameshift	Reported	2005	Premature termination; complete loss of function	Replication slippage	(27–29)
10	TAA→CAA	Stop→Gln	Rare reported	2008	Produces extended, unstable protein	Point mutation under drug pressure	(32–34)
11	Δ380–390 (11 bp)	Large frameshift (nonfunctional)	Similar reported	2005	Large deletion removes critical catalytic residues; complete loss of function	Large-scale replication error	(27,29,30)
12	Δ8 bp near position 446	Frameshift (truncated protein)	Reported	2005	Removes multiple codons in catalytic region; complete loss of enzyme activity	Replication error in repetitive	(29,30,31)
13	TAG→CAG	Stop→Gln	Rare reported	2008	Stop codon loss; extended translation produces nonfunctional protein with misfolded C-terminus	Spontaneous point mutation; rare but recurrent	(32,35,36,37)

Note: (1) Scorpio et al., 1996; (2) Scorpio et al., 1997; (4) Yadon et al., 2017; (5) Li et al., 2021; (7) Mahmood et al., 2022; (8) Tam et al., 2019; (10) Huang et al., 2011; (11) Petrella et al., 2011; (13) Sheen et al., 2009; (14) Xia et al., 2015; (16) Cheng et al., 2000; (18) Sreevatsan et al., 1997; (19) Shenai et al., 2009; (21) Ando et al., 2010; (23) Brossier et al., 2006; (27) Espasa et al., 2005; (29) Spinato et al., 2016; (30) Garnier et al., 2003; (31) Hirano et al., 1997; (32) Huitric et al., 2010; (33) Jureen et al., 2008; (34) Khan et al., 2019; (35) Koser et al., 2013; (36) Louw et al., 2009; (37) Maningi et al., 2015; (38) Maruri et al., 2012.

in MTB (Scorpio & Zhang, 1996; Scorpio et al., 1997). Scorpio and Zhang (1996) first established the critical link between *pncA* mutations and PZA resistance, demonstrating that alterations in this gene encoding PZase/nicotinamidase directly compromise the conversion of the prodrug PZA to its active form, POA. The present findings are consistent with this established mechanism and provide additional support for its relevance within the MDR-TB isolates examined in this study.

Comparable mutation frequencies have also been reported in broader geographic settings, where pncA mutations account for approximately 72–97% of PZA-resistant strains (Miotto et al., 2014; Ramirez-Busby & Valafar, 2015). Xia et al. (2015) reported similar findings in Zhejiang, China, where phenotypic and genotypic characterization revealed high concordance between *pncA* mutations and PZA resistance (Xia et al., 2015). The remaining 15% of our MDR-TB isolates without detectable *pncA* mutations may harbor

resistance mechanisms involving other genetic determinants, such as mutations in the *rpsA* gene or alterations in drug efflux systems (Gopal et al., 2019; Shi et al., 2011). Zimic et al. (2012) demonstrated that POA efflux rate serves as a better proxy for PZA resistance, suggesting that mechanisms beyond *pncA* mutations contribute to the resistance phenotype (Zimic et al., 2012).

4.2. Diversity and distribution of mutation types

Our analysis revealed considerable heterogeneity in the types and locations of mutations within the *pncA* gene across the MDR-TB isolates. This genetic diversity is characteristic of *pncA*-mediated PZA resistance, as the gene exhibits remarkable mutational polymorphism with no single predominant mutation (Cheng et al., 2020; Huang et al., 2003). Unlike other anti-TB drug resistance genes such as *rpoB* (rifampicin resistance) or *katG* (isoniazid resistance), which often display hotspot mutations, *pncA* mutations are distributed throughout the entire gene sequence (Coll et al., 2015; Scorpio et al., 1997).

The *pncA* mutations identified in this study were highly diverse, encompassing missense, nonsense, frameshift, and deletion mutations (Table 4). These alterations were distributed across the entire gene without clustering in any specific hotspot region, reflecting the diverse mechanisms by which *pncA* function can be compromised. Unlike other drug resistance genes, *pncA* shows no single dominant mutation hotspot (Table 4), suggesting multiple independent genetic pathways to PZA resistance. The frequency of mutations in the *pncA* gene varies widely and may be influenced by the geographic origin of the clinical isolates. In this study, substitution, insertion, deletion, and stop-codon mutations were observed at frequencies of 35%, 15%, 15%, and 20%, respectively (Table 5). Yadon et al. (2017) conducted a comprehensive characterization of *PncA* polymorphisms and identified over 400 distinct variants that confer PZA resistance, demonstrating the extensive mutational landscape of this gene (Yadon et al., 2017). Each mutation type impacts PZase enzyme function differently: missense mutations may alter critical amino acid residues in the active site or affect protein stability, nonsense mutations introduce premature stop codons leading to truncated nonfunctional proteins, and frameshift mutations disrupt the reading frame resulting in aberrant protein sequences (Lemaitre et al., 2001; Petrella et al., 2011). Detailed explanations of the effects of *pncA* gene mutations on PZase enzyme function, which may contribute to the development of PZA resistance, are presented in Table 6. The information summarized in this table encompasses not only *pncA* mutants identified in the present study but also previously reported mutants from other studies.

The wide distribution of mutations across the *pncA* gene poses significant challenges for rapid molecular diagnostics. Unlike targeted detection methods that can identify specific hotspot mutations, comprehensive sequencing approaches are required to detect the full spectrum of *pncA* variants (Chakravorty et al., 2015, Miotto et al., 2017). This complexity underscores the importance of whole-gene sequencing for accurate prediction of PZA resistance in clinical settings.

4.3. Structural and functional implications of pzase mutations

The mutations identified in our MDR-TB isolates have important implications for PZase enzyme structure and function. PZase is a metalloenzyme that catalyzes the hydrolysis of PZA to POA, the active form that disrupts *M. tuberculosis* membrane transport and energetics (Zhang & Mitchison, 2003; Zhang et al., 2003). Zhang et al. (2003) elucidated the mode of action of PZA, demonstrating that POA accumulation leads to the disruption of membrane potential and inhibition of membrane transport, ultimately resulting in bacterial cell death.

The three-dimensional structure of PZase reveals a conserved catalytic triad and metal-binding site essential for enzymatic activity (Junqueira-Kipnis et al., 2003; Zhang et al., 2008). Zhang et al. (2008) characterized the structural features of *M. tuberculosis* nicotinamidase/PZase, identifying key residues involved in substrate binding and catalysis (Zhang et al., 2008). Mutations affecting these critical regions would be expected to abolish or significantly reduce enzyme activity, preventing PZA activation and conferring resistance. Conversely, mutations in less conserved regions may have variable effects on enzyme function, potentially explaining the phenotypic variability observed among PZA-resistant strains (Mphahlele et al., 2008; Yadon et al., 2017).

Interestingly, some *pncA* mutations may affect not only enzyme activity but also protein stability, folding, or expression levels (Sheen et al., 2009). Computational studies have shown that certain mutations destabilize the PZase protein structure, leading to misfolding or degradation (Junaid et al., 2020). This multifaceted impact of mutations on protein function highlights the complexity of genotype–phenotype relationships in PZA resistance.

4.3. Clinical implications for MDR-TB management

The high prevalence of *pncA* mutations in our MDR-TB isolates has important clinical implications for treatment regimens. PZA is a cornerstone drug in both drug-susceptible and drug-resistant TB treatment regimens due to its unique sterilizing activity against semi-dormant bacilli (Mitchison, 1985; WHO, 2024a). The WHO guidelines emphasize the importance of PZA in shorter MDR-TB regimens, where it contributes to improved treatment outcomes and reduced duration of therapy (Nunn et al., 2008; WHO, 2022).

However, the presence of *pncA* mutations and consequent PZA resistance in 85% of our MDR-TB isolates suggests that a substantial proportion of patients may not benefit from PZA-containing regimens. This finding underscores the critical need for routine PZA DST in MDR-TB cases (Kwak et al., 2020; Piubello et al., 2014). Traditional phenotypic DST for PZA is technically challenging due to the requirement for acidic pH conditions and prolonged incubation times (Huang et al., 2011). Molecular methods targeting *pncA* mutations offer a more rapid and reliable alternative, enabling timely optimization of treatment regimens (Pholwat et al., 2014; Zaw et al., 2018).

The identification of specific *pncA* mutations in clinical isolates can inform individualized treatment strategies. For patients harboring PZA-resistant strains, alternative drugs with sterilizing activity, such as bedaquiline and pretomanid, or high-dose isoniazid (in the absence of high-level *katG* mutations), may be considered (Conradie et al., 2020; Sotgiu et al., 2016). The WHO consolidated guidelines on TB now recommend personalized treatment regimens based on comprehensive drug susceptibility profiles, including PZA resistance status (WHO, 2024b).

4.4. Geographic and epidemiological considerations

The mutation patterns observed in our study may reflect the specific epidemiological context and transmission dynamics of TB in our region. Geographic variation in *pncA* mutation profiles has been documented globally, with certain mutations showing regional predominance (Stoffels et al., 2012; Sulistyowati et al., 2022). This geographic clustering may result from clonal expansion of specific MDR-TB strains or reflect independent emergence of mutations under selective pressure from PZA use (Casali et al., 2014).

Understanding the local epidemiology of PZA resistance is crucial for public health interventions and infection control measures. High rates of *pncA* mutations in MDR-TB isolates suggest ongoing transmission of drug-resistant strains and highlight the need for enhanced case detection, contact tracing, and implementation of directly observed therapy (DOT) programs (Dheda et al., 2017; Falzon et al., 2016). Furthermore, surveillance of *pncA* mutation patterns can inform regional treatment guidelines and help predict treatment outcomes (Bastos et al., 2016).

4.5. Molecular mechanisms and evolution of PZA resistance

The diverse mutation landscape observed in our study raises important questions about the evolutionary dynamics of PZA resistance in *M. tuberculosis*. The absence of a dominant mutation hotspot in *pncA* contrasts with the mutation patterns seen in other drug resistance genes and suggests that multiple genetic pathways can lead to PZA resistance (Comas et al., 2012). This genetic flexibility may reflect the relatively low fitness cost associated with *pncA* mutations, allowing resistant strains to persist and transmit efficiently (Doustdar et al., 2009; Gagneux et al., 2006).

Zhang and Mitchison (2003) described the curious characteristics of PZA, noting its unique pharmacological properties and the complex relationship between drug concentration, pH, and bactericidal activity (Zhang & Mitchison, 2003). The multifactorial nature of PZA action may explain why diverse mutations throughout the *pncA* gene can confer resistance, as complete loss of PZase activity through various genetic alterations achieves the same phenotypic outcome (Raynaud et al., 1999). The co-occurrence of *pncA* mutations with resistance determinants for other first-line drugs (rifampicin and isoniazid) in our MDR-TB isolates suggests sequential acquisition of resistance mutations or transmission of preexisting MDR strains (Cohen et al., 2014; Maruri et al., 2012). Whole-genome sequencing studies have revealed complex evolutionary pathways in MDR-TB development, with PZA resistance often emerging as a secondary event following rifampicin and isoniazid resistance (Eldholm et al., 2018; Manson et al., 2017).

4.6. Limitations and future directions

Several limitations of this study should be acknowledged. First, the sample size of 20 clinical isolates, while providing valuable insights, may not fully represent the diversity of *pncA* mutations in the broader MDR-TB population. Larger, multicenter studies are needed to comprehensively characterize regional and global mutation patterns. Second, our study focused exclusively on *pncA* gene sequencing and did not investigate other potential mechanisms

of PZA resistance, such as *rpsA* mutations or efflux pump alterations. Future studies should employ whole-genome sequencing approaches to identify additional resistance determinants in *pncA* wild-type but phenotypically resistant strains (Koser et al., 2013). Third, while we identified numerous *pncA* mutations, we did not perform functional validation studies to directly assess their impact on PZase enzyme activity. In vitro enzyme assays and structural modeling studies would provide valuable mechanistic insights into how specific mutations affect protein function (Gopal et al., 2016). In addition, phenotypic drug susceptibility testing was not performed in parallel with genotypic analysis, limiting our ability to establish definitive genotype–phenotype correlations.

Future research should focus on developing rapid, point-of-care molecular diagnostics that can detect the full spectrum of *pncA* mutations in resource-limited settings where TB burden is the highest (Lawn & Nicol, 2011). Advanced sequencing technologies, including next-generation sequencing and nanopore sequencing, offer promising platforms for comprehensive resistance profiling (Nimmo et al., 2020; Votintseva et al., 2017). Integration of molecular diagnostics with clinical decision support systems could facilitate real-time treatment optimization and improve patient outcomes (Dooley et al., 2020).

Furthermore, investigation of compensatory mutations that may restore fitness in PZA-resistant strains would enhance our understanding of resistance evolution and transmission dynamics (Borrell et al., 2013). Longitudinal studies tracking mutation emergence during treatment could identify risk factors for acquired PZA resistance and inform strategies to prevent resistance development (Warner et al., 2025).

5. CONCLUSIONS

This study provides important molecular characterization of the PZase drug target protein in MDR *M. tuberculosis* clinical isolates. Among the PZA resistance phenotypes in 20 MDR-TB isolates studied, 85% had mutations in the *pncA* gene. Analysis of *pncA* mutations in these isolates, the types of mutations in the gene are diverse, including substitutions, deletions, and insertions of N bases. A total of seven MDR-TB isolates experienced base substitution mutations in the *pncA* gene, changing the amino acid for the PZase protein. A total of two isolates had base insertions in the *pncA* gene, and three isolates had deletion mutations that caused frameshift of the PZase protein. Furthermore, four isolates had stop codon mutations to nonstop codons. All of these *pncA* gene mutations are closely involved in the emergence of drug resistance traits in the studied isolates. Among the observed substitution mutations, C175T, G289A, C83A, T173G, and G356T resulted in amino acid changes Leu59Phe, Gly97Ser, Ala28Asp, Phe58Cys, and Trp119Leu in the PZase protein respectively. These substitutions are not listed in the WHO catalogue of MTB drug resistance–associated mutations nor reported in major global *pncA* mutation databases and published systematic reviews, suggesting that they represent previously unreported variants. The statement of the *pncA* mutation triggers PZA resistance. Further studies are needed to confirm the structure–function of the PZase protein it encodes, so that the details of the basis of its resistance can be explained in more depth.

MANDATORY DISCLOSURE ON USE OF ARTIFICIAL INTELLIGENCE

A small portion of translation checking in this paper was assisted using ChatGPT.

AUTHOR CONTRIBUTIONS

Purkan Purkan was responsible for research concept and design, data analysis and interpretation, writing of the article, and the final approval of the article; Nastiti Intan did research concept and design, collection and/or assembly of data, and data analysis and interpretation; Permata Sari was concerned with research concept and design, collection and/or assembly of data, and data analysis and interpretation; Anjar Tri Wibowo did collection and/or assembly of data, data analysis and interpretation, and writing of the article; Wiwin Retnowati was concerned with collection and/or assembly of data, and writing of the article; M Kaisar Sutomo Ramadhan did collection and/or assembly of data, and writing of the article; Iman Permana Maksum was responsible for research concept and design, and data analysis and interpretation; Muhammad Iqbal did critical revision of the article, and the final approval of the article; Loeki Enggar Fitri did critical revision of the article; R Merlyn Sujatha was concerned with data analysis and interpretation, critical revision of the article, and final approval of the article.

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CONFLICT OF INTEREST

All authors have no competing interests.

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REFERENCES

Ando, H., Mitarai, S., Kondo, Y., Suetake, T., Sekiguchi, J.I., Kato, S., et al. 2010. Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan. Clinical Microbiology and Infection. 16(8), 1164–1168. https://doi.org/10.1111/j.1469-0691.2009.03078.x

Bastos, M.L., Lan, Z., & Menzies, D. 2016. An updated systematic review and meta-analysis for treatment of multidrug-resistant tuberculosis. European Respiratory Journal. 49(3), 1600803. https://doi.org/10.1183/13993003.00803-2016

Borrell, S., Teo, Y., Giardina, F., Streicher, E.M., Klopper, M., Feldmann, J., et al. 2013. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. Evolution, Medicine, and Public Health. 2013(1), 65–74. https://doi.org/10.1093/emph/eot003

Brossier, F., Veziris, N., Truffot-Pernot, C., Jarlier, V., & Sougakoff, W. 2006. Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of Mycobacterium tuberculosis with low- and high-level resistance. Journal of Clinical Microbiology. 44(10), 3659–3664. https://doi.org/10.1128/JCM.01054-06

Casali, N., Nikolayevskyy, V., Balabanova, Y., Harris, S.R., Ignatyeva, O., Kontsevaya, I., et al. 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nature Genetics. 46(3), 279–286. https://doi.org/10.1038/ng.2878

Chakravorty, S., Aladegbami, B., Burday, M., Levi, M., Marras, S.A., Shah, S., et al. 2015. Rapid universal identification of bacterial pathogens from clinical cultures by using a novel sloppy molecular beacon melting temperature signature technique. Journal of Clinical Microbiology. 53(4), 1275–1285. https://doi.org/10.1128/JCM.03292-14

Cheng, S.J., Thibert, L., Sanchez, T., Heifets, L., & Zhang, Y. 2000. *pncA* mutations as a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis: Spread of a monoresistant strain in Quebec, Canada. Antimicrobial Agents and Chemotherapy. 44(3), 528–532. https://doi.org/10.1128/AAC.44.3.528-532.2000

Cohen, K.A., Bishai, W.R., & Pym, A.S. 2014. Molecular basis of drug resistance in *Mycobacterium tuberculosis*. Microbiology Spectrum. 2(3), MGM2-0036-2013. https://doi.org/10.1128/microbiolspec.MGM2-0036-2013

Cole S.T., Brosch R., Parkhill J., Garnier T., Churcher C., Harris D., et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 393(6685):537–44.

Coll, F., McNerney, R., Preston, M.D., Guerra-Assunção, J.A., Warry, A., Hill-Cawthorne, G., et al. 2015. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Medicine. 7(1), 51. https://doi.org/10.1186/s13073-015-0164-0

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., et al. 2012. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nature Genetics. 44(1), 106–110. https://doi.org/10.1038/ng.1038

Conradie, F., Diacon, A.H., Ngubane, N., Howell, P., Everitt, D., Crook, A.M., et al. 2020. Treatment of highly drug-resistant pulmonary tuberculosis. New England Journal of Medicine. 382(10), 893–902. https://doi.org/10.1056/NEJMoa1901814

Cronan M.R. 2022. In the thick of it: Formation of the tuberculous ranuloma and its effects on host and therapeutic responses. Frontiers in Immunology. 13, 820134. https://doi.org/10.3389/fimmu.2022.820134

Dheda, K., Gumbo, T., Maartens, G., Dooley, K.E., McNerney, R., Murray, M., et al. 2017. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. The Lancet Respiratory Medicine 5(4), 291–360. https://doi.org/10.1016/S2213-2600(17)30079-6

Dooley, K.E., Miyahara, S., von Groote-Bidlingmaier, F., Sun, X., Hafner, R., Rosenkranz, S.L., et al. 2020. Early bactericidal activity of different isoniazid doses for drug resistant tuberculosis (INHindsight): A randomized, open-label clinical trial. American Journal of Respiratory and Critical Care Medicine. 201(11), 1416–1424. https://doi.org/10.1164/rccm.201910-1960OC

Doustdar, F., Khosravi, A.D., & Farnia, P. 2009. Mycobacterium tuberculosis genotypic diversity in pyrazinamide-resistant isolates of Iran. Microbial Drug Resistance. 15(4), 251–256. https://doi.org/10.1089/mdr.2009.0004

Eldholm, V., Norheim, G., von der Lippe, B., Kinander, W., Dahle, U.R., Caugant, D.A., et al. 2014. Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. Genome Biology. 15(11), 490. https://doi.org/10.1186/s13059-014-0490-3

Espasa, M., Salvadó, M., Vicente, E., Tudó, G., Alcaide, F., Coll, P., et al. 2005. Evaluation of the VersaTrek system compared to the Bactec MGIT 960 system for first-line drug susceptibility testing of Mycobacterium tuberculosis. Journal of Clinical Microbiology. 43(4), 1682–1684. https://doi.org/10.1128/JCM.43.4.1682-1684.2005

Falzon, D., Schünemann, H.J., Harausz, E., González-Angulo, L., Lienhardt, C., Jaramillo, E., et al. 2016. World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update. European Respiratory Journal. 49(3), 1602308. https://doi.org/10.1183/13993003.02308-2016

Gagneux, S., Long, C.D., Small, P.M., Van, T., Schoolnik, G.K., & Bohannan, B.J. 2006. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 312(5782), 1944–1946. https://doi.org/10.1126/science.1124410

Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., et al. 2003. The complete genome sequence of Mycobacterium bovis. Proceedings of the National Academy of Sciences. 100(13), 7877–7882. https://doi.org/10.1073/pnas.1130426100

Gopal, P., Grüber, G., Dartois, V., & Dcoleick, T. 2019. Pharmacological and molecular mechanisms behind the sterilizing activity of pyrazinamide. Trends in Pharmacological Sciences. 40(12), 930–940. https://doi.org/10.1016/j.tips.2019.10.003

Gopal, P., Yee, M., Sarathy, J., Low, J.L., Sarathy, J.P., Kaya, F., et al. 2016. Pyrazinamide resistance is caused by two distinct mechanisms: Prevention of coenzyme A depletion and loss of virulence factor synthesis. ACS Infectious Diseases. 2(9), 616–626. https://doi.org/10.1021/acsinfecdis.6b00070

Hirano, K., Takahashi, M., Kazumi, Y., Fukasawa, Y., & Abe, C. 1997. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis. Tubercle and Lung Disease. 78(2), 117–122. https://doi.org/10.1016/S0962-8479(97)90001-0

Huang, T. S., Kunin, C.M., Lee, S.S., Chen, Y.S., Tu, H.Z., & Liaw, Y.S. 2003. Trends in fluoroquinolone resistance of *Mycobacterium tuberculosis* complex in a Taiwanese medical centre: 1995-2003. Journal of Antimicrobial Chemotherapy. 52(6), 1061–1063. https://doi.org/10.1093/jac/dkg478

Huang, W.L., Chi, T.L., Wu, M.H., & Jou, R. 2011. Performance assessment of the GenoType MTBDRsl test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant Mycobacterium tuberculosis. Journal of Clinical Microbiology. 49(7), 2502–2508. https://doi.org/10.1128/JCM.00197-11

Huitric, E., Verhasselt, P., Koul, A., Andries, K., Hoffner, S., & Andersson, D.I. 2010. Rates and mechanisms of resistance development in Mycobacterium tuberculosis to a novel diarylquinoline ATP synthase inhibitor. Antimicrobial Agents and Chemotherapy. 54(3), 1022–1028. https://doi.org/10.1128/AAC.01611-09

Jankute, M., Cox, J.A., Harrison, J., & Besra, G.S. 2015. Assembly of the mycobacterial cell wall. Annual Review of Microbiology. 69, 405–423. https://doi.org/10.1146/annurev-micro-091014-104121

Junaid, M., Liao, Y., Hoque, M., Akter, W., Khatun, M.S., Ismail, A.M., et al. 2020. Computational study of pyrazinamide resistance conferred by *pncA* mutations in *Mycobacterium tuberculosis*. ACS Omega. 5(46), 30147–30160. https://doi.org/10.1021/acsomega.0c04669

Junqueira-Kipnis, A.P., Kipnis, A., Jamieson, A., Juarrero, M.G., Diefenbach, A., Raulet, D. H., et al. 2003. NK cells respond to pulmonary infection with *Mycobacterium tuberculosis*, but play a minimal role in protection. Journal of Immunology, 171(11), 6039-6045. https://doi.org/10.4049/jimmunol.171.11.6039

Jureen, P., Werngren, J., Toro, J.C., & Hoffner, S. 2008. Pyrazinamide resistance and *pncA* gene mutations in Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy. 52(5), 1852–1854. https://doi.org/10.1128/AAC.00110-08

Khan, M.T., Malik, S.I., Ali, S., Masood, N., Nadeem, T., Khan, A.S., et al. 2019. Pyrazinamide resistance and mutations in *pncA* among isolates of Mycobacterium tuberculosis from Khyber Pakhtunkhwa, Pakistan. BMC Infectious Diseases. 19(1), 116. https://doi.org/10.1186/s12879-019-3764-2

Khawbung J.L., Durbba Nath, Supriyo Chakraborty. 2021. Drug resistant tuberculosis: A review. Comparative Immunology, Microbiology and Infectious Diseases. 74, 101574. https://doi.org/10.1016/j.cimid.2020.101574

Koser, C.U., Bryant, J.M., Becq, J., Török, M.E., Ellington, M.J., Marti-Renom, M.A., et al. 2013. Whole-genome sequencing for rapid susceptibility testing of *M. tuberculosis*. New England Journal of Medicine. 369(3), 290–292. https://doi.org/10.1056/NEJMc1215305

Kwak, N., Choi, S.M., Lee, J., Park, Y.S., Lee, C.H., Lee, S.M., et al. 2020. Diagnostic accuracy and turnaround time of the Xpert MTB/RIF assay in routine clinical practice. PLoS ONE. 15(4), e0231753. https://doi.org/10.1371/journal.pone.0231753

Lawn, S.D., & Nicol, M.P. 2011. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiology. 6(9), 1067–1082. https://doi.org/10.2217/fmb.11.84

Lemaitre, N., Callebaut, I., Frenois, F., Jarlier, V., & Sougakoff, W. 2001. Study of the structure-activity relationships for the pyrazinamidase (*PncA*) from *Mycobacterium tuberculosis*. Biochemical Journal. 353(Pt 3), 453–458. https://doi.org/10.1042/0264-6021:3530453

Li, K., Yang, Z., Gu, J., Hu, W., Zhou, Y., Zhang, J., et al. 2021. Characterization of *pncA* mutations and prediction of PZA resistance in Mycobacterium tuberculosis clinical isolates from Chongqing, China. Frontiers in Microbiology. 11, 594171. https://doi.org/10.3389/FMICB.2020.594171

Louw, G.E., Warren, R.M., Gey van Pittius, N.C., McEvoy, C.R., Van Helden, P.D., & Victor, T.C. 2009. A balancing act: Efflux/influx in mycobacterial drug resistance. Antimicrobial Agents and Chemotherapy. 53(8), 3181–3189. https://doi.org/10.1128/AAC.01577-08

Mahmood, N., Bhatti, S., Abbas, S.N., Hussain, M., Hanif, F., Tahir, M., et al. 2022. The *pncA* gene mutations of Mycobacterium tuberculosis in multidrug-resistant tuberculosis. Biotechnology and Applied Biochemistry. 69(3), 1144–1154. https://doi.org/10.1002/BAB.2278

Maningi, N.E., Daum, L.T., Rodriguez, J.D., Mphahlele, M., Peters, R.P., Fischer, G.W., et al. 2015. Improved detection of Mycobacterium tuberculosis *pncA* mutations by using a molecular beacon assay. Journal of Clinical Microbiology. 53(2), 579–584. https://doi.org/10.1128/JCM.02439-14

Manson, A.L., Cohen, K.A., Abeel, T., Desjardins, C.A., Armstrong, D.T., Barry, C.E., 3rd, et al. 2017. Genomic analysis of globally diverse *Mycobacterium tuberculosis* strains provides insights into the emergence and spread of multidrug resistance. Nature Genetics. 49(3), 395–402. https://doi.org/10.1038/ng.3767

Maruri, F., Sterling, T.R., Kaiga, A.W., Blackman, A., van der Heijden, Y.F., Mayer, C., et al. 2012. A systematic review of gyrase mutations associated with fluoroquinolone-resistant Mycobacterium tuberculosis and a proposed gyrase numbering system. Journal of Antimicrobial Chemotherapy. 67(4), 819–831. https://doi.org/10.1093/jac/dkr566

Ministry of Health, Republic of Indonesia. 2023. *Laporan Penanggulangan Tuberkulosis*. Kemenkes RI. https://www.tbindonesia.or.id/wp-content/uploads/2024/12/Laporan-Program-Penanggulangan-TBC-2023_Final.pdf

Miotto, P., Cabibbe, A.M., Feuerriegel, S., Casali, N., Drobniewski, F., Rodionova, Y., et al. 2014. Mycobacterium tuberculosis pyrazinamide resistance determinants: A multicenter study. *mBio.* 5(5), e01819–14. https://doi.org/10.1128/mBio.01819-14

Miotto, P., Tessema, B., Tagliani, E., Chindelevitch, L., Starks, A.M., Emerson, C., et al. (2017). A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. European Respiratory Journal. 50(6), 1701354. https://doi.org/10.1183/13993003.01354-2017

Mitchison, D.A. 1985. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 66(3), 219–225. https://doi.org/10.1016/0041-3879(85)90040-6

Mphahlele, M., Syre, H., Valvatne, H., Stavrum, R., Mannsåker, T., Mendum, T.A., et al. 2008. Pyrazinamide resistance among South African multidrug-resistant Mycobacterium tuberculosis isolates. Journal of Clinical Microbiology. 46(10), 3459–3464. https://doi.org/10.1128/JCM.00973-08

Nimmo, C., Brien, K., Millard, J., Grant, A.D., & Padayatchi, N. 2020. Bedaquiline resistance in drug-resistant tuberculosis HIV co-infected patients. European Respiratory Journal. 55(3), 1902383. https://doi.org/10.1183/13993003.02383-2019

Nunn, A.J., Phillips, P.P.J., Meredith, S.K., Chiang, C.Y., Converse, P.J., Dalai, D., et al. 2008. A trial of a shorter regimen for rifampin-resistant tuberculosis. New England Journal of Medicine. 380(13), 1201–1213. https://doi.org/10.1056/NEJMoa1811867

Petrella, S., Gelus-Zietal, N., Maudry, A., Laurans, C., Boudjelloul, R., & Sougakoff, W. (2011). Crystal structure of the pyrazinamidase of Mycobacterium tuberculosis: insights into natural and acquired resistance to pyrazinamide. PLoS ONE. 6(1), e15785. https://doi.org/10.1371/journal.pone.0015785

Pholwat, S., Stroup, S., Foongladda, S., & Houpt, E. 2014. Digital PCR to detect and quantify heteroresistance in drug resistant *Mycobacterium tuberculosis*. *PLoS ONE*. 9(7), e99569. https://doi.org/10.1371/journal.pone.0099569

Piubello, A., Harouna, S.H., Souleymane, M.B., Boukary, I., Morou, S., Daouda, M., et al. (2014). High cure rate with standardised short-course multidrug-resistant tuberculosis treatment in Niger: No relapses. The International Journal of Tuberculosis and Lung Disease. 18(10), 1188–1194. https://doi.org/10.5588/ijtld.13.0075

Purkan, P., Hadi, S., Retnowati, W., Sumarsih, S., Wahyuni, D.K., Piluharto, B., et al. 2024. Exploring of pyrazinamidase recombinant activity from PZA-sensitive and resistant Mycobacterium tuberculosis expressed in Escherichia coli BL21 (DE3). Brazilian Journal of Biology. 84. https://doi.org/10.1590/1519-6984.278911

Ramirez-Busby, S.M., & Valafar, F. 2015. Systematic review of mutations in pyrazinamidase associated with pyrazinamide resistance in Mycobacterium tuberculosis clinical isolates. Antimicrobial Agents and Chemotherapy. 59(9), 5267–5277. https://doi.org/10.1128/AAC.00204-15

Raynaud, C., Lanéelle, M.A., Senaratne, R.H., Draper, P., Lanéelle, G., & Daffé, M. 1999. Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiology. 145(Pt 6), 1359–1367. https://doi.org/10.1099/13500872-145-6-1359

Scorpio, A., & Zhang, Y. 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nature Medicine. 2(6), 662–667. https://doi.org/10.1038/nm0696-662

Scorpio, A., Lindholm-Levy, P., Heifets, L., Gilman, R., Siddiqi, S., Cynamon, M., et al. (1997). Characterization of *pncA* mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy. 41(3), 540–543. https://doi.org/10.1128/AAC.41.3.540

Sheen, P., Ferrer, P., Gilman, R.H., López-Llano, J., Fuentes, P., Valencia, E., et al. 2009. Effect of pyrazinamidase activity on pyrazinamide resistance in Mycobacterium tuberculosis. Tuberculosis. 89(2), 109–113. https://doi.org/10.1016/j.tube.2009.01.004

Shenai, S., Rodrigues, C., & Mehta, A. 2009. Time to review the role of pyrazinamide in the treatment of tuberculosis. International Journal of Infectious Diseases. 13(5), 557–561. https://doi.org/10.1016/j.ijid.2008.09.013

Shi, W., Zhang, X., Jiang, X., Yuan, H., Lee, J.S., Barry, C.E., et al. 2011. Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. Science. 333(6049), 1630–1632. https://doi.org/10.1126/science.1208813

Sotgiu, G., Tiberi, S., D'Ambrosio, L., Centis, R., Alffenaar, J.W., Caminero, J.A., et al. 2016. WHO recommendations on shorter treatment of multidrug-resistant tuberculosis. The Lancet Infectious Diseases. 16(10), 1110–1112. https://doi.org/10.1016/S1473-3099(16)30307-7

Spinato, J., Boivin, É., Bélanger-Trudelle, É., Fauchon, L., Tremblay, C., & Soualhine, H. 2016. Genotypic characterization of drug resistant Mycobacterium tuberculosis in Quebec, 2002-2012. BMC Microbiology. 16(1), 164. https://doi.org/10.1186/S12866-016-0786-4

Sreevatsan, S., Pan, X., Zhang, Y., Kreiswirth, B.N., & Musser, J.M. 1997. Mutations associated with pyrazinamide resistance in *pncA* of Mycobacterium tuberculosis complex organisms. Antimicrobial Agents and Chemotherapy. 41(3), 636–640. https://doi.org/10.1128/AAC.41.3.636

Stoffels, K., Mathys, V., Fauville-Dufaux, M., Wintjens, R., & Bifani, P. 2012. Systematic analysis of pyrazinamide-resistant spontaneous mutants and clinical isolates of *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 56(10), 5186–5193. https://doi.org/10.1128/AAC.05385-11

Sulistyowati, T., Soedarsono, & Mertaniasih, N.M. 2022. Mutant vary region of *pncA* gene sequence of pyrazinamide resistance among multidrug resistant Mycobacterium tuberculosis isolates. Journal of Clinical Microbiology and Infectious Diseases. 2(1), 6–10. https://doi.org/10.51559/jcmid.v2i1.18

Tam, K.K.G., Leung, K.S.S., Siu, G.K.H., Tam, L.K.C., Lee, K.C., Cheng, V.C.C., et al. 2019. Direct detection of pyrazinamide resistance in Mycobacterium tuberculosis by use of *pncA* PCR sequencing. Journal of Clinical Microbiology. 57(8), e00145–19. https://doi.org/10.1128/JCM.00145-19

Tobin EH & Tristram D. 2024, Tuberculosis Overview. StatPearls Publishing https://www.ncbi.nlm.nih.gov/books/NBK441916/

Votintseva, A.A., Bradley, P., Pankhurst, L., Del Ojo Elias, C., Loose, M., Nilgiriwala, K., et al. 2017. Same-day diagnostic and surveillance data for tuberculosis via whole-genome sequencing of direct respiratory samples. Journal of Clinical Microbiology. 55(5), 1285–1298. https://doi.org/10.1128/JCM.02483-16

Warner, D.F., Barczak, A.K., Gutierrez, M.G., & Mizrahi, V. 2025. *Mycobacterium tuberculosis* biology, pathogenicity and interaction with the host. Nature Reviews Microbiology, Advance online publication. https://doi.org/10.1038/s41579-025-01201-x

WHO (2022). WHO consolidated guidelines on tuberculosis: Module 4: treatment– Drug-resistant tuberculosis treatment, 2022 update. Geneva: World Health Organization. https://www.who.int/publications/i/item/9789240063129

WHO. 2024a. *Global Tuberculosis Report 2024*. Geneva: World Health Organization. https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2024

WHO. 2024b. WHO consolidated guidelines on tuberculosis. Module 4: Treatment— Drug-susceptible tuberculosis treatment (5th ed.). Geneva: World Health Organization. https://www.who.int/publications/i/item/9789240089998

Xia, Q., Zhao, L.L., Li, F., Fan, Y.M., Chen, Y.Y., Wu, B.B., et al. 2015. Phenotypic and genotypic characterization of pyrazinamide resistance among multidrug-resistant Mycobacterium tuberculosis isolates in Zhejiang, China. Antimicrobial Agents and Chemotherapy. 59(3), 1690–1695. https://doi.org/10.1128/AAC.04541-14

Yadon, A.N., Maharaj, K., Adamson, J.H., Lai, Y.P., Sacchettini, J.C., Ioerger, T.R., et al. 2017. A comprehensive characterization of *PncA* polymorphisms that confer resistance to pyrazinamide. Nature Communications. 8(1), 588. https://doi.org/10.1038/s41467-017-00721-2

Zaw, M.T., Emran, N.A., & Lin, Z. 2018. Mutations inside rifampicin-resistance determining region of rpoB gene associated with rifampicin-resistance in *Mycobacterium tuberculosis*. Journal of Infection and Public Health. 11(5), 605–610. https://doi.org/10.1016/j.jiph.2018.04.005

Zhang, H., Deng, J.Y., Bi, L.J., Zhou, Y.F., Zhang, Z.P., Zhang, C.G., et al. 2008. Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase. The FEBS Journal. 275(4), 753–762. https://doi.org/10.1111/j.1742-4658.2007.06241.x

Zhang, Y., & Mitchison, D. 2003. The curious characteristics of pyrazinamide: A review. The International Journal of Tuberculosis and Lung Disease. 7(1), 6–21.

Zhang, Y., Wade, M.M., Scorpio, A., Zhang, H., & Sun, Z. 2003. Mode of action of pyrazinamide: Disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. Journal of Antimicrobial Chemotherapy. 52(5), 790–795. https://doi.org/10.1093/jac/dkg446

Zimic, M., Fuentes, P., Gilman, R.H., Gutiérrez, A.H., Kirwan, D., & Sheen, P. 2012. Pyrazinoic acid efflux rate in *Mycobacterium tuberculosis* is a better proxy of pyrazinamide resistance. Tuberculosis. 92(1), 84–91. https://doi.org/10.1016/j.tube.2011.09.002