Journal of Zoonotic Diseases
https://jzd.tabrizu.ac.ir/
Published by the University of Tabriz
Online ISSN: 2717-2910

Pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from northwest Iran and neighboring countries

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Article type:
Original article

Keywords:
Pyrazinamide
Tuberculosis
Drug resistance
Northwest Iran

Article history:
Received:
June 9, 2025
Revised:
July 14, 2025
Accepted:
September 3, 2025
Available online:
September 5, 2025

Tuberculosis (TB) is a treatable disease that affects both humans and animals. Pyrazinamide (PZA), a first-line anti-TB drug with potent sterilizing activity, shortens treatment duration, yet its resistance patterns in northwest Iran remain underexplored. This descriptive cross-sectional study, conducted at the Tuberculosis and Lung Diseases Research Center in Tabriz, evaluated PZA resistance among 100 *Mycobacterium tuberculosis* isolates from 50 Iranian and 50 foreign patients (from Nakhchivan Autonomous Republic and the Republic of Azerbaijan) referred for diagnosis and treatment between September 2014 and June 2019. Antimicrobial susceptibility testing (AST) was performed using the proportion method on Löwenstein-Jensen (LJ) medium for isoniazid (INH), rifampin (RIF), ethambutol (EMB), and streptomycin (STM), and Middlebrook 7H10 agar for PZA. Among 100 isolates, 60 (60%) were resistant to at least one of the first-line drugs. Of these, 18 (30%) were resistant to PZA. Out of the PZA-resistant strains, only one isolate was resistant exclusively to PZA, and the rest were resistant to at least one of the main drugs (INH, RIF, STM, and EMB). Out of the 60 strains, 21 (35%) were multidrug-resistant (MDR). Of these, 12 (57%) were resistant to PZA. Of the 18 PZA-resistant strains, 13 (72.2%) were isolated from foreign patients ($P < 0.05$). No PZA resistance was observed in isolates from East Azerbaijan province. These findings indicate a strong association between PZA resistance and MDR-TB, particularly in foreign patients, underscoring the need for routine PZA susceptibility testing, including *pncA* gene sequencing, and enhanced screening in border regions like Tabriz to control drug-resistant TB spread.

Introduction

The *Mycobacterium* (*M.*) genus includes two significant human pathogens: *M. tuberculosis*, which is primarily responsible for tuberculosis (TB), and *M. leprae*, the bacterium that causes leprosy (1). TB is a common disease among humans and animals, and throughout history, various drugs have been used to treat it (2). TB is still a serious infectious disease and ranks second after HIV/AIDS in terms of mortality (3). The World Health Organization (WHO) has reported that in 2023, more than 1.25 million people globally died from TB (4). The

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https://doi.org/10.22034/jzd.2025.20303
https://jzd.tabrizu.ac.ir/article_20303.html
Cite this article: Moaddab S.R. and Roshdi Maleki M. Pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from northwest Iran and neighboring countries. Journal of Zoonotic Diseases, 2025, X

incidence of TB in Iran was 16 cases per 100,000 people in 2015. It decreased to 14 cases per 100,000 people during 2016-2017 and reached 13 cases per 100,000 people in 2020 (5). In East Azerbaijan province, between 5-10 cases per 100,000 people have been reported (6). Although essential drugs such as INH and RIF have been used for almost 50 years, TB remains an infectious disease-related mortality and morbidity (7). Pyrazinamide (PZA), a pyrazine-2-carboxamide analog of nicotinamide, was synthesized in 1936, but its anti-TB effect was discovered in 1952 (8). Before the 1970s, PZA was used as a second-line drug in cases of resistant or relapsed TB due to its high dosage and hepatotoxicity. However, extensive research and clinical experiences have shown that PZA, with its sterilizing ability as a vital component, is one of the most effective anti-TB drugs (9). PZA), a critical first-line anti-TB drug, effectively shortens treatment duration from 9–12 months to 6 months. It is combined with INH and RIF and has been shown to have a high sterilizing effect. PZA is used for the first two months of TB treatment, as taking it for a longer period does not provide any additional benefit (10). Recent efforts to find optimal drug combinations with new drugs to shorten TB treatment in a murine model have shown that PZA is the only drug that cannot be omitted, as otherwise the efficacy of the treatment would be reduced, confirming the unique importance of PZA (11-13). Indeed, the emergence of PZA-resistant *M. tuberculosis* strains is a serious clinical and public health challenge. The essential role of PZA in the treatment of TB highlights the need for accurate and rapid detection of its resistance (3). PZA is converted to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase), which is encoded by the *pncA* gene in *M. tuberculosis*. Mutations in the *pncA* gene are the most common and important cause of resistance to PZA (9, 14, 15). It is more effective on intracellular and dormant bacilli under acidic conditions (16-18). Drug-resistant tuberculosis (DR-TB) is a significant public health problem worldwide, particularly MDR-TB. MDR refers to strains that are resistant to at least two of the most effective anti-TB drugs: INH and RIF (19, 20). In developed, developing, and underdeveloped countries, MDR strains have become a global crisis and disaster, so it is extremely important to detect PZA sensitivity in *M. tuberculosis* strains (21)**.** Since PZA can exhibit its maximum effect at low pH, determining drug resistance is difficult due to the possibility of reduced bacterial growth *in vitro* (22). That is why most mycobacteriology laboratories avoid performing drug susceptibility testing of PAZ as a routine test. For technical reasons, the reports about resistance in TB cases often include four drugs: INH, RIF, EMB, and STM (23, 24). Due to the limited information on PZA resistance in northwest Iran, the present study was conducted to investigate this resistance among *M. tuberculosis* strains.

Materials and methods

Study design and sample collection

This descriptive cross-sectional study was conducted on *M. tuberculosis* isolates collected from patients referred to the Tuberculosis and Lung Diseases Research Center in Tabriz between September 2014 and June 2019. In this study, the resistance of 100 *M. tuberculosis* strains to PZA was evaluated. Of these, 50 strains were isolated from foreign patients (from the Nakhichevan Autonomous Republic and the Republic of Azerbaijan) and 50 strains from Iranian patients at the Tuberculosis and Lung Disease Research Center in Tabriz. All strains were isolated from sputum samples.

Identification of M. tuberculosis strains

Standard conventional laboratory tests like Ziehl-Neelsen staining, niacin production, nitrate reduction, catalase, and pigment production were used to isolate and identify *M. tuberculosis* strains (25).

Drug susceptibility testing for first-line drugs

Susceptibility testing was performed on Löwenstein–Jensen medium for INH, RIF, EMB, and STM by the indirect proportion method. The critical concentration of drugs was 0.2 µg/ml, 40 µg/ml, 2 µg/ml, and 4 µg/ml

for INH, RIF, EMB, and STM, respectively. Susceptibility tests of the mentioned drugs are one of the routine tests for many laboratories at the TB control centers (26, 27).

2.4. PZA susceptibility testing

For the PZA susceptibility test, the indirect proportion method was used with Middlebrook 7H10 agar. First, 10.5 g of Middlebrook 7H10 agar was added to 450 ml of water, then 3.3 g of monopotassium phosphate, 0.5 g of hydrolyzed casein, and 25 ml of glycerol were added to the solution. The solution was sterilized by autoclaving, and after cooling to 54° C, the OADC (oleic acid–albumin–dextrose–catalase) supplement was added and poured into McCartney tubes. To prepare the drug-containing medium, pure PZA powder was dissolved in distilled water and sterilized with a 0.22 μm filter, then added to the medium to adjust the final concentration to 100 μg/ml and pH to 5.9 (22). The bacterial suspension was prepared in Middlebrook 7H9 broth using glass beads. After preparing dilutions of 10^{-2} and 10^{-5}, the suspensions were inoculated onto the culture medium. The results were evaluated after 28 to 42 days by comparing bacterial growth in the drug-free medium (negative control) and the medium containing PZA (positive control). Resistance percentage was calculated using the relevant formula by the amount of bacterial growth [(number of colonies on the drug-containing medium/ number of colonies on medium without the drug) ×100]. When bacterial growth is greater than or equal to 1% of the bacillary population, the strain is considered resistant to the drug (28-30).

Quality Control

For quality control of tests, the standard *M. tuberculosis* strain *H37Rv* (fully susceptible to all drugs) was used.

Statistical Analysis

For statistical analyses, the Pearson's chi-squared and Fisher's exact tests were used to compare the rate of PZA resistance between MDR and non-MDR strains, as well as to compare the number of MDR strains isolated from foreign versus Iranian patients, using SPSS version 22.

Results

The results of this study showed that out of 100 *M. tuberculosis* strains, 60 (60%) were resistant to at least one of the first-line anti-TB drugs. Among them, 38 strains (63.3%) were isolated from Iranian patients and 22 strains (36.7%) from foreign patients (referred to the Tuberculosis and Pulmonary Diseases Research Center, Tabriz University of Medical Sciences). Among the 60 resistant strains, 18 (30%) were resistant to PZA, and 21 strains (35%) were identified as MDR-TB, i.e., resistant to at least two main drugs, INH and RIF. The highest rate of PZA resistance was detected among MDR strains, with 57% (12/21) showing resistance. Of these 12, 9 strains (75%) were from foreign patients and 3 (25%) from Iranian patients living in neighboring provinces. Additionally, 31 strains (51.7%) were identified as mono-resistant, and 8 strains were classified as non-MDR, among which 3 strains (37.5%) were resistant to PZA. A summary of PZA-related resistance patterns is presented in Table 1. Statistical analysis showed no significant difference in the rate of PZA resistance between male and female patients ($P = 0.693$). Among the 18 PZA-resistant strains, only one strain (5.5%) was sensitive to all other first-line drugs. Overall, the findings of this study showed that the level of PZA resistance in foreign patients was significantly higher than in Iranian patients, and this difference also existed among MDR strains ($P < 0.05$). Table 2 presents the distribution of PZA-resistant strains by resistance type and patient nationality. Of the 18 PZA-resistant strains, 13 (72.2%) were from foreign patients, and five (27.8%) were from Iranian patients in neighboring provinces. No PZA-resistant strains were identified among samples from East Azerbaijan province, Iran (Table 2).

Table 1. Factors (gender, nationality, resistance to anti-TB drugs) related to PZA resistance among 60 *M. tuberculosis* strains resistant to at least one of the main drugs (INH, RIF, EMB, STM).

Variable	PZA-Resistant (n=18)	PZA-Susceptible (n=42)	Total (N=60) Percent	*P*-value
Sex				
Male	10	21	31 (51.7%)	0.693
Female	8	21	29 (48.3%)	
Nationality				
Iranian patients	5	33	38 (63.3%)	0.001*
Foreign patients	13	9	22 (36.7)	
Mono-resistant (n=31)				
INH ^{R} (Yes)	1	3	4 (6.7%)	0.821
(No)	17	39	56 (93.3%)	
RIF ^{R} (Yes)	1	4	5 (8.3%)	0.610
(No)	17	38	55 (91.7%)	
STM ^{R} (Yes)	1	20	21 (35%)	0.001*
(No)	17	22	39 (65%)	
MDR (n=21)				
MDR (INH+RIF) ^{R} (Yes)	12	9	21 (35%)	0.001*
(No)	6	33	39 (65%)	
Non-MDR (n=8)				
(INH+EMB) ^{R} (Yes)	1	-	1 (1.7%)	0.123
(No)	17	42	59 (98.3%)	
(INH+STM) ^{R} (Yes)	2	3	5 (8.3%)	0.610
(No)	16	39	55 (91.7%)	
(EMB+STM) ^{R} (Yes)	-	1	1 (1.7%)	0.123
(No)	18	41	59 (98.3%)	
(INH+EMB+STM) ^{R} (Yes)	1	-	1 (1.7%)	0.123
(No)	17	42	59 (98.3%)	

INH (Isonazid), RIF (Rifampin), STM (Streptomycin), EMB (Ethambutol), R (Resistant), and MDR (Multidrug-resistant). *$P < 0.05$ (Pearson chi-square test).

Table 2. Distribution of PZA-resistant *M. tuberculosis* strains by resistance type, nationality, and region of origin (n = 18)

Resistance type	PZA-resistant n/%	Iranian Patients		Foreign Patients (Nakhchivan/Republic of Azerbaijan) (n=13)
		East Azerbaijan	Neighboring provinces of Iran (n=5)	
MDRs (n=21)	12/21 (57%)	0	3	9
Non-MDRs (n=8)	3/8 (37.5%)	0	1	2
Mono-resistant (n=31)	3/31 (9.7%)	0	1	2
Sensitive strains (n=40)	0/40 (0.0%)	0	0	0
Total (n=100)	18/100 (18%)	0	5/18 (27.8%)	13 (72.2%)

MDR: resistant to at least INH and RIF. Foreign patients: from the Nakhchivan Autonomous Republic and the Republic of Azerbaijan. *$P < 0.05$, Pearson's chi-square test.

Discussion

Despite advances in the treatment of TB, DR-TB, especially MDR-TB, has become a life-threatening problem. Major treatment of TB is performed by using drugs: INH, RIF, EMB, STM, and PZA. Since PZA is one of the drugs that is so effective in shortening the time required for the TB treatment to 6 months, determining its susceptibility is so vital and important (17, 31). Studies show that PZA resistance has been part of the overall challenges in treating MDR-TB (32). Studies have shown that using Middlebrook 7H10 agar is a reliable method for detecting PZA-resistant strains (23, 29, 33). Therefore, 7H10 agar was used in the present study. Global reports of drug susceptibility of *M. tuberculosis* are limited to approximately four major first-line drugs and often do not include resistance to PZA.

The use of Middlebrook 7H10 agar in this study allowed the identification of PZA-resistant strains. In the present study, conducted at the Tuberculosis and Pulmonary Diseases Research Center in Tabriz, 100 *M. tuberculosis* strains, all strains isolated from sputum samples, were evaluated for PZA susceptibility. Among 100 isolates, 40 isolates (40%) were sensitive to all drugs, and 60 isolates (60%) were resistant to at least one of the first-line drugs. Among 40 isolates susceptible to INH, RIF, EMB, and STM, one isolate (2.5%) was resistant to PZA. PZA, a unique anti-TB agent with potent sterilizing activity that plays a key role in shortening the TB treatment (9, 31), exhibited a 30% (18/60) resistance rate. Given the potent sterilizing role of PZA, resistance to this drug can be associated with an increased risk of disease relapse, prolonged treatment duration, and even treatment failure in patients. Therefore, rapid identification of PZA resistance is of critical clinical importance.

The most important mechanism of PZA resistance in *M. tuberculosis* is a mutation in the *pncA* gene, which is responsible for producing the PZase (34). Therefore, given the strong correlation between *pncA* gene mutations and phenotypic resistance to PZA (34), the use of molecular tests to identify these mutations could play an important role in the early detection of resistant cases.

The present study showed that the PZA resistance rate in monoresistant strains was 9.7% (3 out of 31). Nasr Esfahani et al. (35) selected 47 drug-resistant *M. tuberculosis* isolates, including 9 MDR, 25 INH-resistant, and 12 RIF-resistant, to evaluate the frequency of resistance to PZA. Their results showed that 19 isolates (40%) were resistant to PZA. In comparison, our study identified 18 out of 60 drug-resistant isolates (30%) as PZA-resistant, reflecting a 10% lower resistance rate. Similar to most studies, the frequency of PZA resistance was high among MDR isolates in their study (7 out of 9, 78%), which is notably higher than in ours (57%). This difference may be attributed to their selection of exclusively drug-resistant isolates. However, they concluded that the rate of PZA resistance among MDR isolates in Isfahan was high. Moreover, among the 19 PZA-resistant isolates, 16 isolates (84.2%) had mutations in the *pncA* gene, indicating the importance of this gene in the development of PZA resistance.

Notably, over 72% of PZA-resistant strains were isolated from foreign patients ($P < 0.05$), whereas no PZA-resistant strains were identified in samples from East Azerbaijan province, Iran ($P < 0.05$). This finding aligns with the high burden of MDR-TB reported in Central Asian countries, including the Republic of Azerbaijan (36, 37). As demonstrated by the present study and previous studies (35, 38-41), PZA resistance is frequently associated with MDR-TB. In contrast, the absence of PZA-resistant strains in East Azerbaijan ($P < 0.05$) may be attributed to lower MDR-TB prevalence, more effective treatment surveillance, or genetic differences in local strains.

The incidence of TB in Iran was reported as 13 cases per 100,000 people (5). According to the report of the Tuberculosis and Leprosy Control Department of the Ministry of Health, in East Azerbaijan province, Iran, between 5-10 cases per 100,000 people have been reported (6). However, border areas, including the northwest of the country, face a higher risk of disease transmission due to their proximity to countries with high TB

prevalence, such as Iraq and the Republic of Azerbaijan. Although the incidence of new TB cases in East Azerbaijan province is low compared to other border provinces (6), high PZA resistance among samples from foreign patients referred to Tabriz for diagnosis and treatment may increase the prevalence of DR-TB in the region. Therefore, enhanced screening and targeted control measures are required.

Various studies show that the level of resistance in Iran's neighboring countries, such as Azerbaijan, Armenia, Pakistan, Afghanistan, and Iraq, is two or three times higher than in Iran (36, 37, 42-44). These findings further support the results of our study. Before 2019, the global trend of TB incidence was declining, but with the outbreak of COVID-19, efforts to control the disease were seriously disrupted (45, 46). Following the COVID-19 pandemic, global TB and MDR-TB incidence increased, particularly in high-burden regions (45). Thus, ongoing studies to monitor PZA resistance are critical for effective MDR-TB treatment in border regions like East Azerbaijan, Iran. In a meta-analysis study, Khademi et al. (47) reported resistance of 20% PZA between 2013 and 2020 in various studies conducted among different provinces of Iran. The highest resistance was seen in Hamadan with 40% and the lowest one was related to Razavi Khorasan (0%). Various studies conducted in China have shown that the rate of *M. tuberculosis* resistance to PZA ranges from 38% to 60% across different provinces, which is higher than the rates reported in studies conducted in Iran (48-50).

An extensive investigation in the United States revealed that, over 11 years, across all 50 states, 2% of non-MDR cases, 38% of MDR cases, and 71% of XDR cases were resistant to PZA (51). In samples that were not MDR, as in our study, the rate of resistance to PZA was lower than in MDR samples. XDR-TB is a form of MDR-TB that is also resistant to any fluoroquinolone and at least one second-line injectable drug (SLID), such as amikacin, kanamycin, or capreomycin (52, 53).

The rates of PZA resistance among MDR strains reported from India and Japan were 59% and 53%, respectively (39, 40), which is consistent with the present study. In the study by Bagheri et al. (54), a comprehensive meta-analysis of 72 studies involving 8,701 *M. tuberculosis* isolates reported that the prevalence of PZA resistance among MDR-TB strains ranged from 50% to 70%, which is consistent with our finding. Furthermore, their analysis highlighted the high diagnostic accuracy of *pncA* gene sequencing, with a pooled sensitivity and specificity of 87% and 94.7%, respectively. These findings support the utility of molecular diagnostics, particularly *pncA* sequencing, as a reliable tool for detecting PZA resistance, especially in settings where phenotypic methods are challenging or unavailable.

These similarities are likely not coincidental but rather reflect a global trend in the spread of PZA resistance among MDR-TB strains, as similar studies from various countries have reported comparable patterns of increasing PZA resistance in MDR-TB (ranging from 50% to 78%). However, comparative genetic studies on strains from different regions are needed to confirm this hypothesis and determine whether similar mutations (e.g., in the *pncA* gene) are involved.

In South Africa (39), Moscow (Russia) (41), and South India (38), the prevalence of PZA resistance among MDR cases was reported as 59%, 73% and 78%, respectively. The higher prevalence of PZA resistance in these countries compared to our study (57%) is likely due to a higher MDR-TB burden and more extensive PZA use in treatment regimens in these regions. Genetic diversity in strains and varying treatment policies may also contribute. Research shows that one in six TB cases and over half of all MDR-TB patients globally are resistant to PZA (55). In addition to geographic variations in resistance rates, differences in *pncA* mutation patterns have been observed across regions. For instance, a study in Canada reported the spread of PZA-monoresistant strains with specific *pncA* mutations, suggesting a potential epidemiological role of these mutations in the transmission of resistant strains (56). It may be inferred that PZA resistance develops following MDR resistance. The study by Xia et al. (49) found that PZA-resistant strains are common among MDR-TB cases in China, but no difference was observed between previously treated and new cases. Conversely, in Latvia, consistent with most

studies, PZA resistance was higher in new cases than in treated TB cases (32). Therefore, the reasons for increased PZA resistance among MDR strains and new TB cases warrant further investigation.

Recommendations for future research

. Given the high prevalence of PZA resistance in MDR-TB patients, it is recommended that molecular assays (e.g., sequencing of *pncA*, *rpsA*, and *panD* genes) be conducted to identify PZA resistance, enabling the design of more effective treatment regimens.

. Comparative studies between border and non-border provinces could clarify the role of migration and cross-border transmission of resistant strains.

. Additionally, it should be investigated whether incomplete treatments or non-standard drug prescriptions, particularly in neighboring countries, contribute to PZA resistance, as limited access to adequate treatment remains a challenge in many regions with high MDR-TB prevalence.

Conclusion

In this study, of 100 *M. tuberculosis* isolates, 60% were resistant to at least one first-line drug, and 30% of these isolates (18 out of 60) exhibited resistance to PZA. PZA resistance among MDR-TB strains was 57%, predominantly observed in foreign patients. No cases of PZA resistance were detected in isolates from East Azerbaijan province, Iran. These findings highlight that PZA resistance, particularly among foreign patients and MDR strains, poses a serious concern for public health and TB control policies. Given PZA's critical role in shortening TB treatment duration, accurate assessment of PZA resistance should be a routine component of laboratory testing for suspected MDR-TB cases. Furthermore, in border regions and referral centers like Tabriz, enhanced drug susceptibility screening and continuous monitoring of resistance patterns using precise laboratory methods could significantly contribute to controlling the spread of drug-resistant TB.

Acknowledgment

This work was supported by the Tuberculosis and Lung Disease Research Center of Tabriz University of Medical Sciences and the Student Research Committee.

Ethical approval

This article is an extract from a research project approved by Tabriz University of Medical Sciences with tracking code 25779. This study was conducted on strains isolated from patients referred to the Tuberculosis and Lung Diseases Research Center of Tabriz University of Medical Sciences. No patient identification information was used, and their personal information was not included in the study.

Conflict of interest statement

The authors declare no conflicts of interest.

Artificial Intelligence Statement

The authors declare that artificial intelligence tools were used in a limited manner solely to improve the grammar and clarity of some sentences, but were not used in the preparation of the scientific content of this manuscript. The authors take full responsibility for the scientific integrity and accuracy of the manuscript.

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