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ORIGINAL RESEARCH

Prevalence, Transmission and Genetic Diversity of Pyrazinamide Resistance Among Multidrug-Resistant *Mycobacterium tuberculosis* Isolates in Hunan, China

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Background: China is a country with a burden of high rates of both TB and multidrug-resistant TB (MDR-TB). However, published data on pyrazinamide (PZA) resistance are still limited in Hunan province, China. This study investigated the prevalence, transmission, and genetic diversity of PZA resistance among multidrug-resistant *Mycobacterium tuberculosis* isolates in Hunan province.

Methods: Drug susceptibility testing (DST) with the Bactec MGIT 960 PZA kit and pyrazinamidase (PZase) testing were conducted on all 298 MDR clinical isolates. Moreover, 24-locus MIRU-VNTR and DNA sequencing of *pncA, rpsA*, and *panD* genes were conducted on 180 PZA-resistant (PZA-R) isolates.

Results: The prevalence of PZA resistance among MDR-TB strains reached 60.4%. Newly diagnosed PZA-R TB patients and clustered isolates with identical *pncA, rpsA*, and *panD* mutations showed that transmission of PZA-R isolates played a significant role in the formation of PZA-R TB. Ninety-eight mutation patterns were observed in the *pncA* among 180 PZA-R isolates, and seventy-one (72.4%) were point mutations. Twenty-four of these mutations are new, including 2 base substitutions (V93G and T153S) and 22 nucleotide deletions or insertions. The W119C was found in PZA-S isolates, on the other hand, F94L and V155A mutations were found in both PZA resistant and susceptible isolates with positive PZase activity, indicating that they were not associated with PZA resistance. This is not entirely in line with the WHO catalogue. Ten novel *rpsA* mutations were found in 10 PZA-R isolates, which all combined with mutations in *pncA*. Thus, it is unpredictable whether these mutations in *rpsA* can impact PZA resistance. No *panD* mutation was found in all PZA-R isolates.

Conclusion: DNA sequencing of *pncA* and PZase activity testing have great potential in predicting PZA resistance.

Keywords: tuberculosis, pyrazinamide, transmission, *pncA* mutations, *rpsA* mutations, *panD* mutations, China

Introduction

Drug-resistant tuberculosis (TB), especially multidrug-resistant tuberculosis (MDR-TB, defined as resistance to isoniazid (INH) and rifampicin (RFP)), is still a severe public health threat. Globally in 2021,[1] there were approximately 5.8 million notified new TB cases, 71% (2.4/3.4 million) of people diagnosed with bacteriologically confirmed pulmonary TB were tested for rifampicin resistance (RR), the same level of coverage as in 2020 (2.1/3.0 million) and up from in 2019 (2.2/3.6 million). Among these, 141,953 cases of MDR/RR-TB and 25,038 cases of pre-extensively drug-resistant TB (pre-XDR-TB, defined as TB that is resistant to RFP and any fluoroquinolone) and XDR-TB (resistant to RFP, any fluoroquinolone and at least one of bedaquiline or linezolid) were detected, for a combined total of 166,991. China remains the second highest MDR-TB burden

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country. A national survey of drug-resistant TB in China showed that 5.7% and 25.6% of new cases and retreated cases respectively had MDR-TB, nearly twice the global average MDR-TB,[2] including pre-XDR-TB and XDR-TB, poses a significant challenge to TB therapy and control programs.[3]

Pyrazinamide (PZA) is a critical first-line antituberculosis drug with effectiveness in targeting intracellular and semi-dormant bacilli living in an acidic environment inside macrophages.[4] In clinic, PZA is used at the initial intensive phase of chemotherapy in combination with other first-line drugs, including INH, RFP, and ethambutol (EMB). PZA can shorten the treatment regimen for drug-susceptible TB and can also increase the success rate of the MDR-TB treatment.[5] Despite the important role of PZA in TB treatment, PZA resistance in MTB has increased in TB cases. The estimated global burden of new PZA-resistant TB cases annually is 1.4 million cases, of which 270,000 cases occur in MDR-TB patients.[6]

Currently, the automated Bactec MGIT 960 system is the commonly used phenotypic assay for PZA susceptibility testing. However, false resistance to PZA by this method has been often reported by previously studies, which demonstrated that high *M. tuberculosis* inoculum may cause loss of PZA activity by increasing the pH of the medium.[7,8] In addition, pyrazinamidase (PZase) activity test, known as Wayne's method, can also be used as a screening method for indirect PZA drug susceptibility testing (DST). This method uses orange-red color change of pyrazinoic acid (POA, the active form of PZA catalyzed by the enzyme PZase[9]) to react with ferrous ammonium sulfate.[10] Subsequently, modified Wayne's methods that indirectly measure pyrazinamidase activity via pyrazinoic acid in a liquid medium was reported.[11] However, PZase activity test requires a significant number of clinical *M. tuberculosis* cultures and its sensitivity is lower than that of Bactec MGIT 960 system.[12]

Molecular diagnostic approaches are increasingly being used for prediction of PZA resistance in *M. tuberculosis*. Recently, a novel line probe assay, the Genoscholar PZA-TB II assay (NIPRO Corporation, Japan), has been endorsed by WHO for the detection of PZA resistance in clinical *M. tuberculosis* complex isolates.[13] This assay encompasses the PZase coding gene, *pncA*, and 18 nucleotides upstream. Mutations in *pncA* can affect its enzyme activity and are considered as the major mechanism of PZA resistance. Previous studies showed that the prevalence of *pncA* mutation in PZA-R *M. tuberculosis* complex isolates varied from 24 to 100%.[14–18] In addition, ribosomal protein S1 (RpsA) is critical in protein translation and ribosome-sparing process of trans-translation,[19] and has been reported as a target of POA. However, one recent study found that RpsA interacts with single-strand RNA, but not with POA.[20] Some studies showed that mutations in *rpsA* that coded ribosomal protein S1 was associated with PZA resistance in isolates expressing wild-type *pncA*,[21–23] whilst some studies showed that the frequency of *rpsA* mutations showed no statistical difference between PZA-R and PZA-susceptible (PZA-S) isolates.[24,25] Another gene *panD*, encoding aspartate alpha-decarboxylase, is considered to be a new target of POA. PanD involves the synthesis of β-alanine, which is a precursor for pantothenate and coenzyme biosynthesis. However, previous studies found that POA is a weak PanD enzyme inhibitor.[26,27] The roles of mutations in *panD* in PZA resistance were uncertain. None of mutations in *panD* were detected in a study from Pakistan,[23] whilst only four out of 161 and one out of 11 PZA-R isolates carried mutations in *panD* without that in *pncA* in two studies from China.[21,28] These findings suggest that the mechanism of PZA resistance in *M. tuberculosis* is complex and remains to be investigated further.

On the other hand, molecular epidemiological methods that combine epidemiological investigations and genotyping of *M. tuberculosis* strains provide the means to assess the transmission of TB.[29,30] Due to the critical role of PZA in TB therapy, understanding the transmission patterns of PZA-R TB is crucial to take effective public health measures in controlling such disease. Previous studies have investigated the transmission patterns of PZA-R TB among MDR-TB patients in the mainland China.[21,25,28,31] However, no data is available in Hunan province, which remains a high TB burden province in China.[32]

In this study, we investigated the prevalence of PZA resistance, mutation characteristics of *pncA, rpsA* and *panD* genes in PZA-R isolates, and transmission of PZA-R isolates among MDR-TB in Hunan province. The association between the activity of PZase and *pncA* mutation was also analyzed in this study.

Materials and Methods

Study Population and *M. tuberculosis* Complex Isolates

Our study was conducted in Hunan Chest Hospital, the province-level and largest specialized hospital for TB treatment in Hunan province, China. A total of 737 patients diagnosed as MDR-TB between January 2016 and December 2018 were reviewed (Figure 1). Information including gender, age, treatment history, and results of the phenotypic DST for first-line

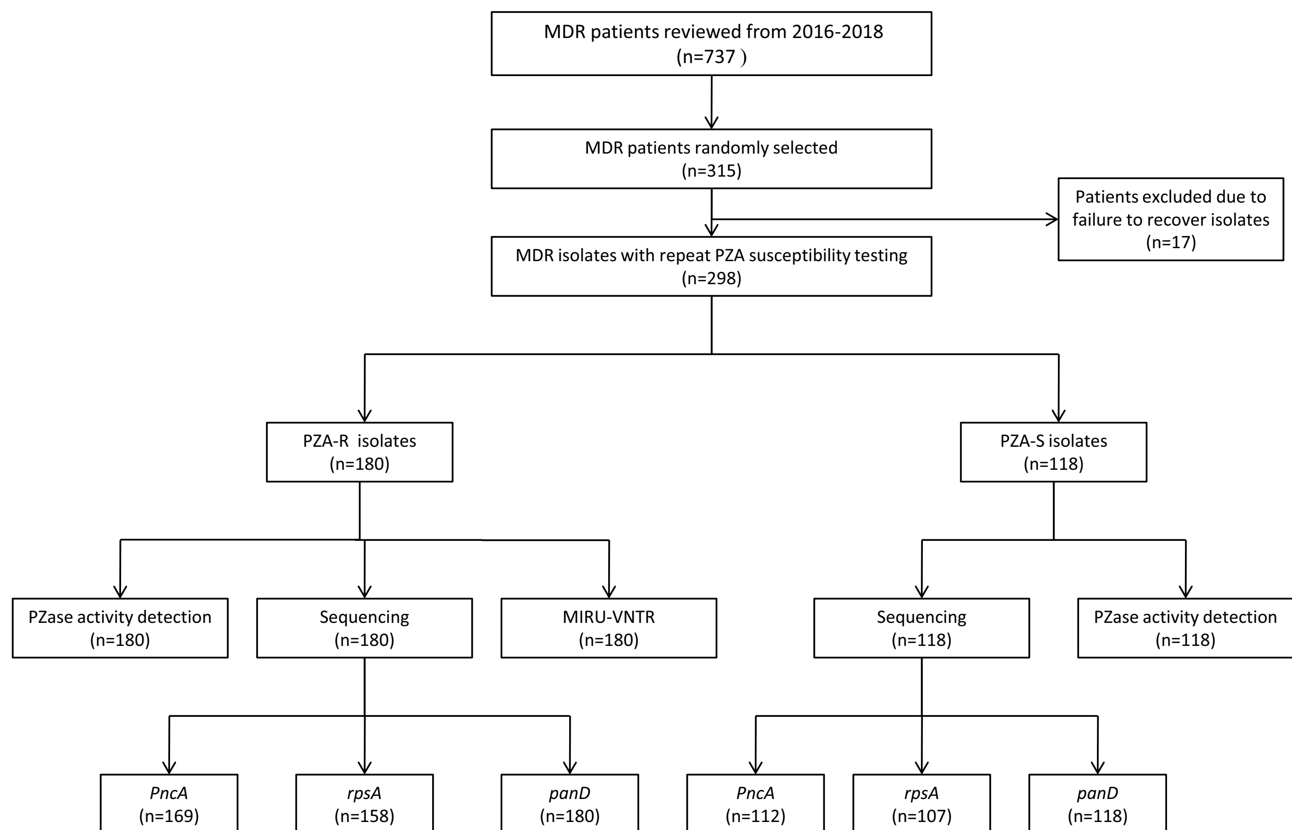

Figure 1 Study outline.
Abbreviation: PZA-R, pyrazinamide-resistant; PZA-S, pyrazinamide-susceptible; PZase, pyrazinamidase.

including isoniazid (INH), rifampicin (RFP), streptomycin (STM), ethambutol (EMB) and PZA, and second-line anti-tuberculosis drugs, was collected. DST for the first-line antituberculosis drugs was carried out using Bactec MGIT 960 SIRE and PZA kits according to the manufacturer's instructions (Becton, Dickinson and Company, Sparks, MD, USA), while DST for the second-line drugs including kanamycin (KM), amikacin (AK), capreomycin (CPM), and levofloxacin (LFX) was performed with the proportion method on Lowenstein-Jensen (L-J) medium. The critical concentrations of first-line and second-line drugs for DST were as follows: STM, 1.0 µg/mL; INH, 0.1µg/mL; RFP, 1.0 µg/mL; EMB, 5.0 µg/mL; PZA, 100.0 µg/mL; KM, 30.0 µg/mL; AK, 30.0 µg/mL; CPM, 40.0 µg/mL; LFX, 2.0 µg/mL.[33,34]

We randomly selected 315 cases from 737 MDR-TB patients for further analysis. There were no statistical differences between the selected and unselected cases, in terms of patients' gender, age and TB treatment history. All 315 patients' isolates were preserved at −80°C before use. Finally, excluding 17 isolates failed to grow on L-J solid medium, a total of 298 isolates were included in the study.

MGIT960 Pyrazinamide Susceptibility Testing, Pyrazinamidase Activity Detection and Pyrazinamide MIC Test

PZA susceptibility testing using Bactec MGIT 960 PZA kits was repeated on the recovered isolates to confirm PZA resistance. The inoculum was prepared from bacterial growth on Lowenstein-Jensen solid medium. After incubating at 36 ± 1°C for 21 days, the fresh colonies were scraped as many as possible using a sterile 10 µL loop, then were added to a sterile screw cap bottle containing glass beads moistened by 100 µL 0.05% Tween 80. Bacterial clumps were dispersed by vortexing for 15-20 seconds and then were suspended in 3 mL saline solution. Thereafter, the suspension was left to settle by standing for 10-15 minutes and the upper 0.5 mL suspension was transferred to a new tube for bacterial concentration measurement. The bacterial concentration was adjusted to a McFarland turbidity of 0.5 and then diluted in sterile saline (1:5 dilution). This diluted inoculum was used for the Bactec MGIT 960 PZA DST, which were performed according to the manufacturer's instructions

(Becton, Dickinson and Company, Sparks, MD). The critical concentration for PZA was 100.0 µg/mL. *M. tuberculosis* H37Rv (ATCC 27924) was used as PZA susceptible control strain. If the first two rounds of the PZA susceptibility testing results were discordant, the assay was performed again to confirm the phenotype of PZA susceptibility.

The PZase activity testing was carried out through the Wayne's screening method, with slight modifications.[35] Briefly, 7H10 agar (9.5 g) (Difco) was dissolved in 450 mL water, then 2 mL glycerol and PZA (Sigma) at a final concentration of 400 ug/mL were added to make the PZase agar medium, followed by autoclaved at 121 °C for 10 min. Then 5 mL aliquots of this autoclaved PZase agar were distributed in sterilized plastic tubes. Then a heavy loopful of actively growing culture was carefully inoculated on the surface of the PZase agar medium and incubated at 37°C for 4 days. One milliliter of ferrous ammonium sulfate (1%) was added to each tube after incubation and observed for an initial 4 h for the appearance of a pink band (positive) in the subsurface agar. The PZA-susceptible *M. tuberculosis* H37RV was used as a positive control strain and PZA-resistant isolates of *M. tuberculosis* confirmed to be negative by the PZase activity testing earlier were used as negative controls. The PZase assay results were recorded independently by two observers who were not aware of the phenotype of PZA susceptibility results.

For the PZA susceptible isolates that harbored *pncA* mutations classified as linked to PZA resistance[36], the PZA MICs were determined using the BD EpiCenter system by dissolving PZA powder to 25, 50, 100, and 200 µg/mL in MGIT culture medium.

DNA Extraction, PCR Amplification and Sequencing

Fresh cultured bacteria on L-J solid medium were scraped and resuspended in 500 µL of 0.9% saline water. After heat inactivation at 85 °C for 20 min, the cell suspension was incubated in an ultrasonic bath for 15 min at 95 °C. Cells were then centrifuged at 13,000 × g for 5 min, and the supernatant containing DNA was transferred to a fresh tube and stored at −20 °C for further use. The fragments containing *pncA* were amplified using primers according to a previous study:[24] *pncA*-F (5'-TGCCACTCGCCGGTAACCGG-3' (nt 321 to 340 downstream of *pncA*)) and *pncA*-R (5'-GGTGGCCGCC GCTCAGCTGG-3' (nt −119 to −100 of *pncA*)). The fragments containing *rpsA* were amplified using two primer sets as follows: *rpsA*-f1 (5'-GGAGGTGTCGGTGGGCTA-3'), *rpsA*-r1 (5'-CTTCCTGAGTCGCCTTGAGT-3'); *rpsA*-f2 (5'-TCG TCAACTTCGGCGCGTTC-3'), *rpsA*-r2 (5'-GACCACTTCACGCGCCAACA-3'). The fragments containing *panD* were amplified using primers as follows: *panD*-F (5'-GGCTGCTGGACAACATTGC-3') and *panD*-R (5'-GATCG TCAGTGCCAGTTCGT-3'). All PCRs were conducted under the following conditions: 94 °C denaturation for 5 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 60 °C for *pncA* and *rpsA*, or 57 °C for *panD*, and 1 min at 72 °C, with a final extension of 7 min at 72 °C. All the PCR products were sent to Beijing Ribio Biotech Co, Ltd for DNA sequencing using the Applied Biosystems 3730XL DNA sequencer. The sequences were analyzed using GeneDoc (Version 3.2) by comparing with the *M. tuberculosis* H37Rv sequence (GenBank accession no.NC_000962.3).

Mycobacterial Interspersed Repetitive Units-Variable-Number Tandem Repeat Typing and Data Analysis

The 24-locus MIRU-VNTR typing method was carried out using previously reported primers.[37] PCRs for all MIRU-VNTR loci were performed in a reaction volume of 20 µL containing 10 µL 2 × Taq mixture, 2 µL genomic DNA template, and 0.5 µmol of each primer set. The PCR amplification program was 95 °C for 15 min, followed by 40 cycles at 94 °C for 1 min, 59 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. Five microliters of the amplicon was run on a 2.0% agarose gel, with a 100 bp DNA ladder being run every ten lanes. The size of the PCR fragments was defined using the Bio-Rad Quantity One software, version 4.6.2 (BIO-RAD Laboratories, Hercules, CA, USA). The data from 24-locus MIRU-VNTR genotypes were analyzed through BioNumerics software, version 8.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Statistical Analysis

IBM SPSS (version 16.0) (IBM Corporation, Armonk, NY, USA) was used to analyze the data. We used a chi-square test to examine the correlations between demographic characteristics, clinical variables and PZA resistance. Furthermore, Pearson correlation coefficient was used to determine the correlation between PZase activity, PZA susceptibility and *pncA* mutation. A two-sided $P < 0.05$ is considered statistically significant.

Results

Pyrazinamide Resistance Among Multidrug Resistant Isolates

The detailed susceptibility profiles of the 737 MDR isolates to nine drugs (INH, RFP, STM, EMB, PZA, LFX, KM, CPM, and AK) are shown in Table 1. In order to assure the accuracy of PZA resistance, PZA susceptibility testing was repeated on 298 MDR isolates. In total, 45 isolates had discordant PZA susceptibility results. Considering the results of the third round of PZA susceptibility testing, 39 were classified as PZA-susceptible, while the remaining six were classified as PZA-resistant. In total, 180 out of 298 (60.4%) were resistant to PZA.

Factors Associated with Pyrazinamide Resistance

The risk factors associated with PZA resistance are summarized in Table 2. MDR isolates from patients aged 30 to 59 were less likely to produce PZA resistance (OR: 0.424, 95% CI: 0.223~0.806) than those from patients who were less than 30 years old. MDR-TB patients with STM resistance, EMB resistance, or LFX resistance demonstrated a significantly increased risk for developing PZA resistance, with odds ratios (95% CI) of 2.408 (1.452~3.991), 2.558 (1.584~4.130) and 2.245 (1.370~3.680), respectively. Other factors such as gender, treatment history and resistance to

Table 1 Drug Susceptibility Profiles of 737 MDR Isolates

Classification and Drug Susceptibility Profiles	NO. of MDR- Isolates	Proportion (%)
INH+RFP	107	14.5
INH+RFP+EMB	36	4.9
INH+RFP+STM	123	16.7
INH+RFP+STM+EMB	111	15.1
INH+RFP+EMB+AK	2	0.3
INH+RFP+EMB+KM	1	0.1
INH+RFP+EMB+LFX	34	4.6
INH+RFP+LFX	38	5.2
INH+RFP+STM+EMB+AK	1	0.1
INH+RFP+STM+EMB+CPM	1	0.1
INH+RFP+STM+EMB+KM	1	0.1
INH+RFP+STM+EMB+KM+CPM	1	0.1
INH+RFP+STM+EMB+LFX	144	19.5
INH+RFP+STM+KM+CPM	1	0.1
INH+RFP+STM+LFX	84	11.4
INH+RFP+EMB+LFX+CPM	1	0.1
INH+RFP+EMB+LFX+CPM+AK	1	0.1
INH+RFP+EMB+LFX+KM+CPM	4	0.5
INH+RFP+EMB+LFX+KM+CPM+AK	1	0.1
INH+RFP+LFX+AK	1	0.1
INH+RFP+LFX+KM	1	0.1
INH+RFP+LFX+KM+CPM+AK	1	0.1
INH+RFP+STM+EMB+LFX+AK	1	0.1
INH+RFP+STM+EMB+ LFX+KM	4	0.5
INH+RFP+STM+EMB+LFX +KM +AK	6	0.8
INH+RFP+STM+EMB+LFX +KM +CPM	12	1.6
INH+RFP+STM+EMB+LFX +KM +CPM+AK	10	1.4
INH+RFP+STM+LFX+CPM	2	0.3
INH+RFP+STM+LFX+KM	3	0.4
INH+RFP+STM+LFX+KM+CPM	3	0.4
INH+RFP+STM+LFX+KM+CPM+AK	1	0.1
Total	737	

Table 2 Factors Associated with PZA Resistance in MDR-TB Patients

Characteristic	NO. of PZA-S Patients (n=118)	NO.of PZA-R Patients (n=180)	OR (95% CI)	*P* Value
Sex				
Male	95	135	Reference	
Female	23	45	1.377 (0.781–2.427)	0.268
Age group				
<30	16	44	Reference	
30–59	84	98	0.424 (0.223–0.806)	0.009
≥60	18	38	0.768 (0.345–1.711)	0.518
Treatment history				
New	35	40	Reference	
Retreated	83	140	1.451 (0.852–2.471)	0.169
STM resistance				
No	49	41	Reference	
Yes	69	139	2.408 (1.452–3.991)	0.001
EMB resistance				
No	64	57	Reference	
Yes	54	123	2.558 (1.584–4.130)	0.000
LFX resistance				
No	71	72	Reference	
Yes	47	108	2.245 (1.370–3.680)	0.001
KM resistance				
No	113	164	Reference	
Yes	5	16	2.607 (0.835–8.141)	0.088
CPM resistance				
No	116	171	Reference	
Yes	2	9	2.933 (0.611–14.084)	0.160
AK resistance				
No	115	170	Reference	
Yes	3	10	2.195 (0.581–8.296)	0.236

other drugs were not statistically significant among patients infected with PZA-resistant and PZA-susceptible MDR-TB isolates (P>0.05).

Mutations in the *pncA* Gene

Among 180 PZA-R isolates, 169 were successfully sequenced. The results showed that 162 (95.9%) harbored mutations in the *pncA* gene as well as its promoter region (Supplementary Table 1), including 129 isolates with nucleotide substitutions in the promoter or the coding region of *pncA*, and 33 isolates with insertions or deletions. It is of note that ten isolates carried multiple mutations, including six isolates with double mutations and four isolates with triple mutations. Overall, 98 mutation patterns were found here (Supplementary Table 1). The most frequent mutation pattern was the nucleotide substitution from A to G at position −11 (n=13) in the promoter region of *pncA*, followed by V7G in the coding region of *pncA* (n=11). Based on the WHO catalogue,[36] of the 71 base substitution types in the *pncA* gene, 39 (54.9%) were classified as "associated with resistance", followed by 12 (16.9%) as "associated with resistance-interim" and 2 (2.8%) as "not associated with resistance" (Supplementary Table 1). Significantly, 8 (11.3%) base substitution types in the *pncA* gene were regarded as "uncertain significance" (I5T, L35P, F58V, G78D, T100P, D136G, T142P, and V155A). The remaining 10 types (23.2%) were not included in the WHO catalogue, including eight (C14Y, G24R, L27M, V93G, C138Y, T153S, L156R, and A178P) described previously and two (V9S and A28N) detected for the first time (Table 3). In addition, the WHO catalogue omitted 26 nucleotide deletions or insertions with various lengths of

Table 3 *pncA* Mutations Not Included in WHO Catalogue[36] and Classified as "Uncertain Significance" by WHO in PZA-Resistant MDR Strains and Their Pyrazinamidase Activities

Nucleotide change	Amino acid Change(Nucleotide codon change)	No. of isolates	PZase activity	
			Result	No. of isolates
Substitution				
T14C	I5T(ATC→ACC)[c]	2	Negative	2
G41A	C14Y(TGC→TAC)[a]	2	Negative	2
G70C	G24R(GGC→CGC)[a]	1	Negative	1
T104C	L35P(CTG→CCG)[c]	1	Positive	1
T172G	F58V(TTC→GTC)[c]	1	Negative	1
G233A	G78D(GGC→GAC)[c]	1	Positive	1
T278G	V93G(GTG→GGG)[a]	2	Positive	2
A298C	T100P(ACC→CCC)[c]	1	Negative	1
A407G	D136G(GAT→GGT)[c]	2	Negative	2
G413A	C138Y(TGT→TAT)[a]	1	Negative	1
A424C	T142P(ACG→CCG)[c]	1	Negative	1
A457T	T153S(ACC→TCC)[a]	1	Negative	1
T464C	V155A(GTG→GCG)[c]	1	Positive	1
G532C	A178P(GCC→CCC)[a]	1	Negative	1
Deletion				
G68deletion	Frameshift[a,b]	1	Negative	1
G164deletion	Frameshift[a,b]	1	Negative	1
ATCGTC16-21deletion	IV6-7deletion[a,b]	1	Negative	1
CACCGGACTA 182–191deletion	Frameshift[a,b]	2	Negative	2
TCAGCGGTACT 218–228deletion	Frameshift[a,b]	1	Negative	1
G360deletion	Frameshift[a,b]	1	Negative	1
C384deletion	Frameshift[a,b]	1	Negative	1
G385deletion	Frameshift[a,b]	1	Negative	1
TT451-452 deletion	Frameshift[a,b]	1	Negative	1
ATGGTGGACGT-11–1deletion	Promoter[a,b]	1	Negative	1
Insertion				
A193insertion	Frameshift[a]	1	Negative	1
A99insertion	Frameshift[a,b]	2	Negative	1
GG257-258 insertion	Frameshift[a]	1	Negative	1
G315insertion	Frameshift[a,b]	1	Negative	1
T322insertion	Frameshift[a,b]	1	Negative	1
A336insertion	Frameshift[a,b]	1	Negative	1
G392insertion	Frameshift[a]	2	Negative	2
GG392-393 insertion	Frameshift[a]	2	Negative	2
GGT393-395 insertion	V131VV(GTC→GTGGTC)[a,b]	1	Negative	1
GC445-446 insertion	Frameshift[a,b]	1	Negative	1
C457insertion	Frameshift[a,b]	1	Negative	1
ACC505-507 insertion	T169insertion[a,b]	2	Negative	2
TCGACCCGGGTGACCACTTCTCCGGCACACCG GACTATTCCTCGTCGTGGCCACCGCATTGCGT CAGCGGTACTCCCGGCGCGGACTTCCATCCCA GTCTGGACACGTCGGCA268-380 insertion	Frameshift[a,b]	1	Negative	1

(*Continued*)

Table 3 (Continued).

Nucleotide change	Amino acid Change(Nucleotide codon change)	No. of isolates	PZase activity	
			Result	No. of isolates
Multiple mutations				
C79A/G82A/C83A	L27M(CTG→ATG)[a]/ A28N(GCC→AAC)[a,b]	1	Negative	1
G25T/T26C	V9S(GTG→TCG)[a,b]	2	Negative	2
G25T/T26C/T467G	V9S(GTG→TCG)[a,b]/ L156R(CTG→CGG)[a]	1	Negative	1
G304insertion/C305T	Frameshift[a,b]	1	Negative	1
G392insertion/G520T	Frameshift[a,b]	1	Negative	1
T451C/T452A/ T455insertion	Frameshift[a,b]	2	Negative	2

Notes: [a]*pncA* mutations that are not included in WHO catalogue. [b]*pncA* mutations that have not been reported previously. [c]*pncA* mutations that were included in the WHO catalogue and classified as "uncertain significance".
Abbrevition: PZase, pyrazinamidase.

nucleotides in *pncA*, of which 4 were previously reported and 22 were newly reported (Table 3). It is worth noting that seven PZA-resistant isolates harbored wild-type *pncA*.

In contrast, 17 of the 118 PZA-susceptible isolates harbored non-synonymous mutations in the *pncA* gene (Supplementary Table 2), of which four carried mutations (P62T, F94L, W119C, and V180F) were regarded as PZA resistance-associated by WHO.[36] Nonetheless, two isolates with F94L and W119C mutations did show PZA susceptibility since their MIC values ≤100.0 ug/mL in our study and they possessed positive Pzase activity (Table 4).

Correlation of *pncA* Mutations to Pyrazinamidase Activity and PZA Susceptibility

PZase activity testing showed that eight PZA-R isolates harboring *pncA* mutations including L35P, P62L, D63A, G78D, V93G, F94L, and V155A showed positive PZase activity (Supplementary Table 1). We also found six PZA-susceptible isolates that were PZase-negative (Supplementary Table 2). Interestingly, the F94L and V155A mutations were found both in PZA resistant and susceptible isolates with positive PZase activity. The correlation coefficient between PZase activity and *pncA* mutation was 0.828 (P<0.05), while the correlation coefficient between *pncA* mutation and PZA susceptibility was 0.808 (P<0.05).

Mutations in the *rpsA* and *panD* Genes

Ten PZA-resistant isolates harbored novel non-synonymous mutations in the *rpsA* gene which combined with mutations in *pncA*, while four PZA-susceptible isolates carried non-synonymous mutations (Table 5). In addition, a synonymous mutation *rpsA* mutation (A636C) was found in 147 PZA-resistant and 76 PZA-susceptible isolates. In terms of the *panD* gene, non-synonymous mutations were found exclusively in two PZA-susceptible isolates, but not in 180 PZA-resistant isolates. However, seven PZA-resistant isolates did not harbor any non-synonymous mutations in the *pncA, rpsA* or *panD* genes.

Table 4 *pncA* Mutation Patterns, Pyrazinamidase Activity and Pyrazinamide MIC Results in Four Pyrazinamide-Susceptible Strains

Strains	Nucleotide change	Amino acid Change (Nucleotide Codon Change)	Final confidence grading[36]	PZase activity	MIC of PZA (ug/mL)
1	C184A	P62T(CCG→ACG)	Assoc w RI	Negative	200
2	T280C	F94L(TTC→CTC)	Assoc w R	Positive	50
3	G357T	W119C(TGG→TGT)	Assoc w R	Positive	100
4	G538T	V180F(GTC→TTC)	Assoc w R	Positive	200

Abbrevition: PZase, pyrazinamidase.

Table 5 *rpsA* and *panD* Mutation Patterns of PZA-Resistant and PZA-Susceptible MDR Strains

Gene	PZA Susce-Ptibility	Nucleotide Change	Amino Acid Change (Nucleotide Codon Change)	No. of isolates	Combined pncA Mutations
rpsA	Resistant	C142G/A636C	R48G(CGG→GGG)[b]	1	V7G(GTC→GGC)
		G151A	V51M(GTG→ATG)[b]	1	A-11G
		A161G/A636C	D54G(GAC→GGC)[b]	1	D8N(GAC→AAC)
		G193A	A65T(GCC→ACC)[b]	1	H57Y(CAC→TAC)
		C227T	P76L(CCC→CTC)[b]	1	A-11G
		T482G/A636C	L161R(CTG→CGG)[b]	1	G385deletion(Frameshift)
		A590C/A636C	E197A(GAG→GCG)[b]	1	T142P(ACG→CCG)
		A636C/G751deletion	Frameshift[b]	1	A171V(GCG→GTG)
		A636C/T761C	V254A(GTT→GCT)[b]	1	L120R(CTG→CGG)
		G997A	V333M(GTG→ATG)[b]	1	Y103Stop(TAC→TAG)
		A636C/G894A	No change	1	—
		A636C	No change	147	—
	Sensitive	C168A/A636C	No change	1	—
		A368C/A636C	D123A(GAC→GCC)	1	No mutation
		C417G/A636C	I139M(ATC→ATG)[b]	1	No mutation
		T494C/A636C	I165T(ATC→ACC)[b]	1	No mutation
		A636C	No change	76	—
		A636C/C1319A	A440E(GCG→GAG)[b]	1	No mutation
		No mutation	—	26	—
		ND	—	11	—
panD	Resistant	No mutation	—	180	—
	Sensitive	C115T	No change	1	—
		C295T	R99C(CGC→TGC)[b]	1	No mutation
		A40G	T14A(ACG→GCG)[b]	1	No mutation

Note: "b": Mutation not previously reported.

Prediction of PZA Resistance by DNA Sequencing and Pyrazinamide Activity Testing

Using MGIT 960 PZA susceptibility testing as the reference method, the detection of mutations in *pncA* showed a sensitivity of 95.9% (95% CI, 91.7%~98.0%) and a specificity of 84.7% (95% CI, 76.8%~90.2%) (Table 6). The sensitivities of sequencing *rpsA* and *panD* were very low, indicating little diagnostic value. Sequencing *pncA* together with *rpsA* or *panD* mutations did not increase the sensitivity of 95.9% (95% CI, 91.7%~98.0%) but the specificity decreased to 74.5% (95% CI, 65.5% ~81.9%). PZase activity testing reached a sensitivity of 96.1% (95% CI, 92.2% ~98.1%) and specificity of 94.9% (95% CI, 89.4% ~97.7%).

Table 6 The Evaluation of the Drug Susceptibility and DNA Sequencing, PZase Activity

DNA Sequencing/ PZase Activity	No. of Resistant Isolates		No. of Susceptible Isolates		Sensitivity (95% CI) (%)	Specificity (95% CI) (%)
	With Mutation/ Negative	Without Mutation/ Positive	With Mutation/ Negative	Without Mutation/ Positive		
Locus						
pncA	162	7	17	94	95.9 (91.7–98.0)	84.7 (76.8–90.2)
rpsA	10	170	4	103	5.6 (3.1–9.9)	96.3 (90.8–98.5)
panD	0	180	2	116	0.0 (0.0–2.1)	98.3 (94.0–99.5)
pncA /*rpsA* /panD	162	7	27	79	95.9 (91.7–98.0)	74.5 (65.5–81.9)
PZase activity	173	7	6	112	96.1 (92.2–98.1)	94.9 (89.4–97.7)

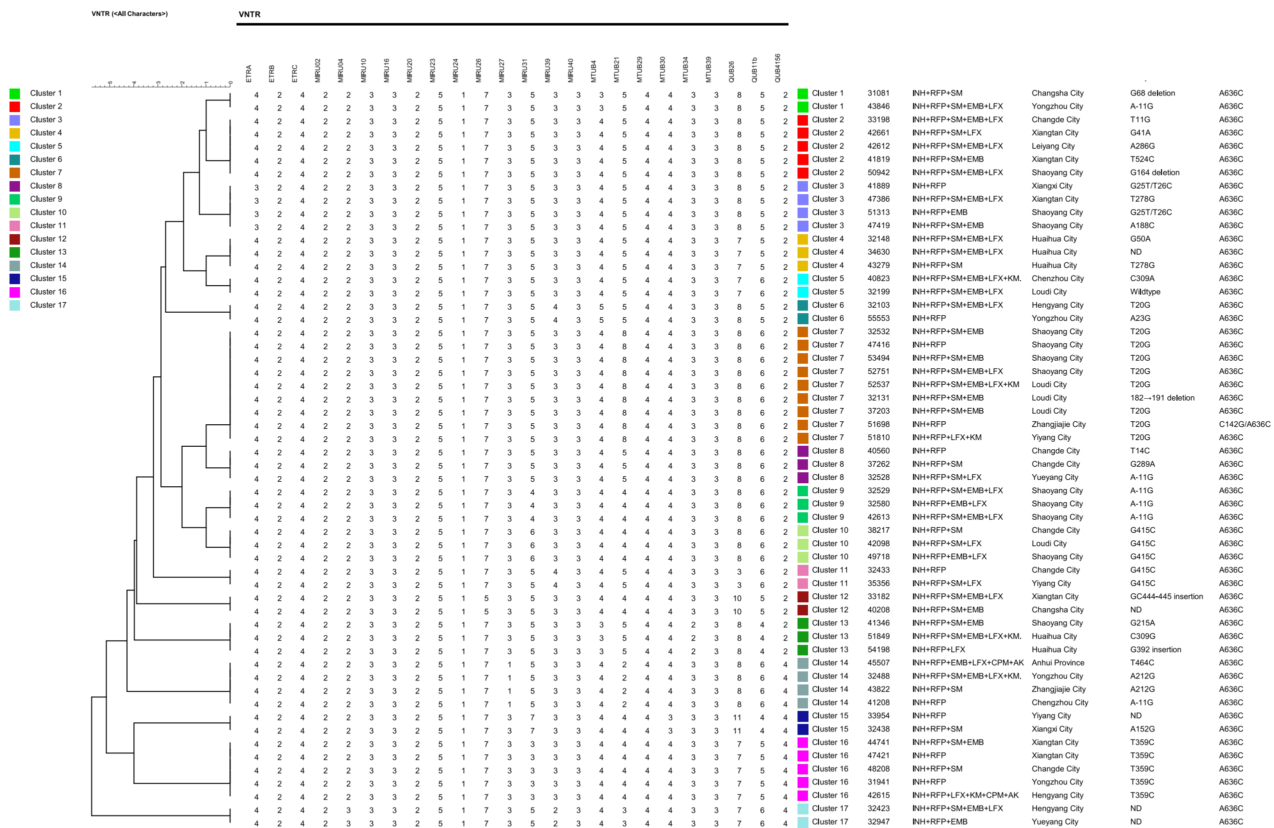

Figure 2 The clustered isolates with *pncA, rpsA* and *panD* mutations. 24 loci VNTR based dendrogram and isolates profiles of 17 clusters in which pncA, rpsA and panD mutant isolates were involved.

Transmission of PZA-R Isolates

Drug resistance in newly diagnosed TB patients is generally considered as primary drug resistance, while drug-resistant TB patients with clustered isolates indicate recent transmission. Among the 180 PZA-R cases, 40 (28.6%) were newly diagnosed TB patients, while 140 (71.4%) were retreated. To identify potential recent transmission of PZA-R isolates during the research period, 180 PZA-R isolates were analyzed by 24-locus MIRU-VNTR typing. The results showed 141 genotypes: 124 (68.9%) isolates had a unique MIRU-VNTR profile and 56 (31.1%) isolates from eight newly diagnosed TB patients and 48 retreated patients were involved in 17 clusters. We further found that seven clusters respectively had at least two isolates with identical *pncA, rpsA* and *panD* mutations. Additionally, the residence information of patients showed that two PZA-R patients in Cluster 2, respectively four and two in Cluster 11 and three in Cluster 13 lived in the same village (Figure 2). These clues suggest the recent transmission of PZA-R *M. tuberculosis*.

Discussion

It is necessary to better understand the transmission of PZA-R tuberculosis and genetic basis of PZA resistance to achieve the goal of ending TB. To the best of our knowledge, this was the first study on the transmission and genetic basis of PZA-resistant *M. tuberculosis* in Hunan province, China. To minimize errors in PZA drug susceptibility testing, we repeated MGIT 960 PZA susceptibility testing on recovered strains to validate the phenotype of PZA resistance. The results showed that 180 out of 298 isolates had true PZA resistance, while the remaining 118 isolates were susceptible to PZA. We further investigated the 180 isolates with PZA resistance.

Our results showed that the proportion of PZA resistance in MDR isolates in Hunan, China was 60.4%, which was at the medium level compared with other regions in Mainland China (47% - 69.02%),[21,25,28,31,38,39] and was higher than those from other countries, including Myanmar (58.9%),[40] Peru[41] (47.7%), and South Korea (31.5%).[42] This suggests that rapid detection of PZA resistance is an urgent need for the control of MDR-TB in the whole of China including

Hunan province. We also analyzed the correlations between PZA resistance and demographic characteristics. The results showed that MDR-TB patients aged 30 to 59 were less likely to produce PZA resistance than those aged less than 30. Furthermore, we also found that PZA resistance was associated with resistance to STM, EMB, and LFX. Previous studies have established the correlation between the resistance of PZA and other anti-tuberculosis drugs.[25,28,38,39] A study from Alame-Emane found that PZA resistance in *M. tuberculosis* arose after RFP and fluoroquinolone resistance,[43] which partly supported our findings on the link between PZA and LFX resistance. Given the correlations of resistance between PZA and other drugs, PZA susceptibility testing is crucial for the proper management of MDR-TB patients with regimens containing PZA.

Our study also showed that 28.6% (40/180) PZA-R MDR isolates were from newly diagnosed TB patients and 31.1% (56/180) PZA-R MDR isolates were clustered, indicating the transmission of drug resistance. In addition, identical types of mutation in *pncA, rpsA*, and *panD* were also detected in the same clustered isolates, and patients whose isolates in the same clusters were from the same living regions. This further indicated recent transmission of PZA-R MDR-TB.

We further investigated the mutations in *pncA, rpsA*, and *panD* genes among 169 PZA-R isolates that were successfully sequenced. Of the 71 types of base substitution in the *pncA* gene, 8 (11.3%) (I5T, L35P, F58V, G78D, T100P, D136G, T142P, and V155A) were classified as "uncertain significance" by the WHO,[36] while 10 types (23.2%) were not included in the WHO catalogue,[36] including two novel mutation types (V9S and A28N) and eight that had been previously reported (C14Y, G24R, L27M, V93G, C138Y, T153S, L156R, and A178P).[16,17,44–49] Base substitutions of C14Y, L27M, C138Y, and L156R were found in the PZA-R resistant isolates in previous studies, and the connection between I5T, G24R, F58V, G78D, T100P, D136G, T142P, V155A, and A178P with PZA resistance is contentious based on findings from previous studies.[13,14,44–49] For substitution mutations in *pncA* classified as "Uncertain significance" and those not included in the WHO catalogue, PZA resistance was predicted using the SUSPECT-PZA webserver.[50] The results showed that *pncA* mutations T100P, T153S, and A178P were predicted as "susceptible", while other mutations were predicted as "resistant". Our study detected 24 *pncA* mutations in PZA-R isolates for the first time, and most of them (91.7%) were deletions or insertions with various lengths of nucleotides.

Unexpectedly, PZase activity testing showed that eight PZA-R isolates harboring *pncA* mutations including L35P, P62L, D63A, G78D, V93G, F94L, and V155A without mutations in *rpsA* and/or *panD*, showed positive PZase activity. This suggests that these mutations may not affect the PZase activity, and other PZA resistance mechanisms may be responsible for these PZA-R isolates. On the other hand, there were 17 PZA-susceptible isolates harboring *pncA* non-synonymous mutations. We further determined the MICs of four PZA-susceptible strains carrying *pncA* mutations (P62T, F94L, W119C, and, V180F) that had been regarded as PZA resistance associated by the WHO.[36] We also discovered that two isolates with mutations of F94L and W119C did show PZA susceptibility (MIC values $\leq$ 100 ug/mL) and possessed positive activity. F94L and V155A mutations with positive PZase activity were found both in PZA resistant and susceptible isolates. This is not completely consistent with the WHO catalogue[36] and suggests that the F94L, W119C, and V155A mutations do not appear to be associated with PZA resistance. It also indicated that drug resistance resulted from interactions of multiple macromolecules in organisms, including genes, transcripts and proteins and so on.[51]

Non-synonymous *rpsA* mutations were detected in four PZA-S isolates, and 10 PZA-R isolates which combined with mutations in *pncA*, thus it is uncertain whether these mutations in *rpsA* can affect PZA resistance. A recent study found that RpsA is not the binding target of POA, the active form of PZA, and concluded that RpsA may not be involved in the mechanism of PZA action in *M. tuberculosis*.[52] While previous studies showed that aspartate decarboxylase PanD emerged as a target of POA[27,53], no non-synonymous mutations were detected in the *panD* of all PZA-R isolates in our study. This means that *panD* mutation may not be associated with PZA resistance. In addition, seven PZA-R isolates did not harbor any mutations in *pncA, rpsA*, and *panD*, indicating that additional unknown mechanisms may be involved in PZA resistance.

The WHO target product profiles for new molecular assays for *M. tuberculosis* require more than 90% sensitivity and 95% specificity.[54] Using MGIT 960 PZA susceptibility testing as reference, *pncA* sequencing for PZA resistance prediction showed sensitivity exceeded 90% and a moderate specificity of 84.7%. However, the mutations found in *rspA* or *panD* did not improve the sensitivity, but reduced the specificity by 10.2%. Although the specificity of *pncA* sequencing was slightly lower in our study, the sensitivity was higher than that in other studies conducted in Mainland China, such as 90.0% (Hangzhou[21]), 77.97% (Zhejiang[25]), 89.52% (Henan[28]), 83.1% (Ningbo[31]), 79.3% (Beijing[39]). Therefore, it is necessary to detect *pncA* mutations to improve PZA resistance detection in clinical practice. We also

evaluated the prediction performance of PZase activity testing. Compared to MGIT 960 PZA drug susceptibility testing, PZase activity testing reached a sensitivity of 96.1% and specificity of 94.9%. The PZase activity testing can be completed within four days, and the equipment and reagents involved in this experiment are affordable and easily accessible. Hence, PZase activity testing can be used as an alternative DST method to predict PZA resistance, particularly for low-income countries.

Limitation of the Study

Our research has several limitations. Firstly, we did not conduct whole-genome sequencing (WGS) on PZA-R isolates. Compared to MIRU-VNTR, WGS has a higher resolution in genotyping and can provide information on the lineage of the clinical MTB isolates, which would enable us to analyze the linkage between PZA resistance and specific strain lineages. Additionally, the mechanism of PZA resistance is complex and involves multiple genes such as *pncA, rpsA* and *panD*. Our study used Sanger sequencing to individually sequence each gene, while WGS can provide information on genes related to PZA resistance at the whole-genome level. Recently, WHO evaluated the application prospects of targeted next-generation sequencing (tNGS) in the diagnosis of drug-resistant tuberculosis. They consider that the available evidence supports the use of targeted NGS to detect drug resistance after TB diagnosis, to guide clinical decision-making for drug-resistant TB treatment. Therefore, we believe that the use of tNGS for the detection of PZA-R isolates has good application prospects in the future.

Conclusion

Given the important role of transmission in the incidence of PZA-R TB, targeted interventions such as PZA-R TB cases management and source of infection finding are urgently needed to prevent further transmission of PZA-R TB in Hunan province. DNA sequencing of *pncA* and PZase activity testing have great potential in predicting PZA resistance. In addition, sequencing data of *rpsA* and *panD* from this study do not support correlation between these two genes and PZA resistance.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was in line with the Declaration of Helsinki and ethically approved (LS2022060701) by the Ethics Committee of Hunan Chest Hospital in 2022. All methods were performed in accordance with the relevant guidelines and regulations. Each patient signed an informed consent form during hospitalization and treatment.

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Disclosure

The authors declare there are no conflicts of interest.

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