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ORIGINAL RESEARCH

Pyrazinamide Resistance and pncA Mutation Profiles in Multidrug Resistant Mycobacterium Tuberculosis

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Purpose: Pyrazinamide (PZA) is a critical component of standardized chemotherapy for tuberculosis (TB) and is recommended for the treatment of multidrug-resistant (MDR) TB. We aimed to characterize mutations in *pncA* of *M. tuberculosis* and evaluate their diagnostic accuracy for PZA susceptibility in China. We also combined genotypic methods with phenotypic susceptibility testing and pyrazinamidase (PZAse) activity to confirm PZA-resistant *M. tuberculosis* isolates.

Results: An evaluation of 82 MDR *M. tuberculosis* strains revealed that 28.0% (23/82) were phenotypically resistant to 100 mg/L PZA and 15.9% (13/82) showed resistance to 300 mg/L PZA. Mutations in *pncA* were detected at 33 unique sites, and the majority were point mutations. No evident mutation hotspots or mutations affecting multiple amino acids were found, but the association between *pncA* mutations and PZA resistance was significant under 100 and 300 mg/L. The sensitivity of *pncA* mutation detection for predicting PZA susceptibility was 82.6% (19/23), and the specificity was 61.0% (36/59), based on 100 mg/L PZA, whereas the sensitivity was 84.6% (11/13) and the specificity was 55.1% (38/69), based on 300 mg/L PZA. All mutations identified in the highly PZA-resistant (300 mg/L) strains had an 80% loss relative to PZAse activity. No evident PZAse activity loss was observed in one synonymous mutation strain and the loss exceed 60% in all other strains.

Conclusion: The association between pncA mutation and PZA resistance was significant. Relatively, the molecular method have shown better reliability than the phenotypic method for the detection of PZA resistance. This provides a theoretical basis for the clinical diagnosis of drug-resistant TB.

Keywords: MDR, PZA, *pncA*, DST, Beijing genotype, enzymatic activity

Introduction

Tuberculosis (TB), a respiratory disease caused by *Mycobacterium tuberculosis* infection, is one of the top 10 health problems worldwide. The World Health Organization (WHO) reported about 9.9 million new cases of TB worldwide in 2020, of which 2.59% exhibited a multidrug-resistant phenotype (MDR-TB). MDR-TB continues to be a significant threat to public health.[1,2]

Pyrazinamide (PZA) is an important first-line anti-TB drug known for its unique sterilizing activity and reduction of TB therapy time.[3–5] Good penetration of PZA into the lung tissue of patients with MDR-TB has been shown to produce a significant correlation between tissue PZA concentrations and necrosis. Tissue penetration of pyrazinamide into the diseased lung and the favorable acidic environment of most chronic tuberculous lesions, which promotes the bactericidal

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and sterilizing activity of pyrazinamide.[6] In drug-susceptible TB, the addition of PZA to the rifampin/isoniazid regimen has facilitated the shortening of the treatment period from 9 to 6 months.[7,8] A recent study showed that introducing a molecular drug susceptibility test (DST) to PZA successfully improved treatment outcomes, increasing the relapse-free success rate to 82.4% in PZA-susceptible cases and shortening the regimen period to 12 months without any additional agents. In addition, clinical trials to evaluate short-course chemotherapy regimens in MDR-TB are ongoing, including STREAM, Simplici TB, MDR-END, and TB-TRUST trials. All these regimens include PZA.[10–16] The potential synergistic activities between PZA and important second-line as well as novel drugs (such as bedaquiline, delamanid, and pretomanid) highlight its importance in both current and future MDR-TB treatment strategies.

The sterilizing activity of PZA is activated following its catalysis by pyrazinamidase (PZAse), and PZA susceptibility evaluations depend on the acidity of the culture medium. Currently, growth-based DSTs using the MGIT 960 PZA kit are widely regarded as the gold standard for evaluating *M. tuberculosis* drug susceptibility to PZA. However, there are limitations to the current PZA resistance tests. The long incubation period for *M. tuberculosis* strains means phenotypic DSTs may take months to complete, resulting in significant diagnostic delays. Moreover, PZA phenotypic DSTs are rarely performed in routine clinical settings as the test is time-consuming and technically demanding. The low-pH medium and stringent inoculum size of these assays often lead to inconsistent and non-reproducible results that deter clinicians from relying on these tests for guidance.

Molecular testing for PZA susceptibility is based on the detection of mutations in the *pncA* gene. The *M. tuberculosis pncA* gene encodes a functional PZAse, the key enzyme in PZA activation, and mutations in this gene may reduce the activity of PZAse and thus increase *M. tuberculosis* resistance to PZA.[17] Several studies have shown that 72–98% of PZA resistance is due to *pncA* mutations, which are highly diverse and scattered across both the open reading frames and upstream regulatory regions of this gene.[18,19] Similarly, some studies have found a correlation between mutations in the *pncA* gene and phenotypic PZA susceptibility, but the performance of these molecular diagnostic assays is not as good as that for isoniazid (INH) and rifampicin (RIF).[19,20]

This study aimed to characterize the mutation pattern and mutant types in the *pncA* gene of MDR-TB strains from Henan, China using direct sequencing and subsequently comparing these results to those of the MGIT 960 PZA susceptibility assay and confirming these results with a PZAse activity test. We also evaluated the agreement between the genotypic and phenotypic evaluations of PZA susceptibility in this MDR *M. tuberculosis* population dominated by Beijing-genotype strains. Our findings will help to further develop molecular PZA detection methods.

Materials and Methods

M. tuberculosis Clinical Strains

From January 2017 to March 2018, a population of 95 MDR *M. tuberculosis* clinical strains were collected via active screening of clinical *M. tuberculosis* isolates from patients in Henan Province. The isolation, culture, and species identification of these clinical strains were conducted in the Tuberculosis Reference Laboratory (TRL) at Henan Provincial Centers for Disease Control and Prevention (Henan CDC), China. This study was approved by the Institutional Review Board of the Institute of Pathogen Biology at the Chinese Academy of Medical Sciences & Peking Union Medical College. Written informed consent was obtained from all patients before bacterial isolation.

Drug Susceptibility Testing (DST)

Drug susceptibility testing was performed with WHO-recommended proportion method. DSTs for INH and RIF were conducted by the TRL at the Henan CDC. They used the Löwenstein–Jensen proportion method recommended by the WHO/IUATLD at the following critical drug concentrations: INH, 0.2 mg/L; RIF, 40.0 mg/L.[21] PZA resistance of these bacilli was evaluated using the BACTEC MGIT 960 automatic method, according to the manufacturer's instructions (Becton Dickinson, Sparks, MD, USA). The MGIT 960 medium is a modified Middlebrook 7H9 broth with a reduced pH of 5.9. According to the BACTEC MGIT 960 PZA instructions, the McFerland turbidity of the strain was adjusted to 1, then a total of 500 μL of each inoculum was added to the PZA-containing tube, and a 1:10 dilution of the inoculum was added to another tube without PZA as the growth control. We evaluated two concentrations of PZA (100 mg/L and

300 mg/L) to define the phenotypic drug resistance of each strain; 100 mg/L is the concentration recommended by the manufacturer and is the common concentration used in routine clinical practice.

Genomic DNA Extraction and Detection of pncA Mutations

The crude genomic DNA of each strain was extracted using the boiling method,[22] and the entire *pncA* coding region was amplified by PCR. PCR was conducted according to the protocols for Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) with some modifications. The reaction mixture contained 10 μL HF buffer, 1.5 μL DMSO, 2 μL dNTP mix (10 nM), 0.5 μL of each primer (final concentration: 50 pmol/L), 0.5 μL Phusion® High-Fidelity DNA Polymerase, and 3 μL crude genomic DNA. The PCR cycling conditions were set up as follows: initial denaturation at 98 °C for 30s; 35 cycles of 98 °C for 10s, 63 °C for 30s, 72 °C for 1 min; and then final extension at 72 °C for 7 min. PCR products (763 bp) were then submitted to Sinogenomax Co. Ltd (Beijing, China) for sequencing. The primers used for amplification were *pncA*-1: 5′-CGAACGTATGGTGGACGTAT-3′ (position −18 to position +2 of *pncA* ORF) and *pncA*-2: 5′-CCGATGAAGGTGTCGTAGAA-3′ (downstream 165 bp to 184 bp of the stop codon of *pncA* ORF); the same primers were also used in the subsequent sequencing of the PCR products.

Sequence Alignment and Statistical Analysis

Sequence data were assembled using by Seqman pro (version11.2, DNAstar Lasergene, Madison, WI, USA), and mutations were then evaluated by comparing these sequences to the *pncA* gene (NC_000962.2) from *M. tuberculosis* H37Rv, the *M. tuberculosis* reference strain. The frequency calculations and association analyses were performed using SPSS for windows (version 26.0; SPSS Inc. Armonk, NY, USA) and a P-value < 0.05 was considered significant.

MIRU-VNTR Genotyping and Strain Identification

MIRU-VNTR genotyping was performed as previously described.[23] Briefly, this method relies on the evaluation of seven VNTR loci (VNTR-7) known for their high discriminatory power in Chinese *M. tuberculosis* strains.[24] Samples with more than one band in the polymerase chain reaction (PCR) products on any VNTR locus were treated as mixed strains and excluded from the evaluation. Most of these strains were identified as the Beijing genotype, and this classification was confirmed using Deletion-targeted Multiplex PCR (DTM-PCR) as previously reported.[25]

Expression and Purification

PZAse for each mutation type containing a carboxy-terminal His-tag was expressed in *Escherichia coli* and purified by affinity chromatography on a Ni-nitrilotriacetic acid column (GE Healthcare, Uppsala, Sweden) as described previously.[26,27] Briefly, the *pncA* gene was amplified, using PCR with specific primers. DNA of an *M. tuberculosis* isolate cloned into a pET28a expression vector to produce a His-tag prokaryotic expression plasmid. After verified by sequencing, this fusion protein was expressed in *E.coli* BL21 cells. After that, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analysis the purity and molecular weight of the proteins, and we have measured protein concentration according to the BCA method.[28]

PZAse Enzymatic Activity

PZAse activity was then evaluated using the standard Wayne test.[29,30] Briefly, PZA (100 μg/mL) was hydrolyzed using 100 ng/μL recombinant PZAse, and pyrazinoic acid (POA) production was quantified following the addition of 20% $FeNH_4(SO_4)_2$, and the reaction was stopped with 0.1 M glycine–HCl (pH 3.4) after 1 min. The absorbance was then measured at 450 nm and the OD was adjusted to produce a standard curve of known concentrations. Each recombinant PZAse was then evaluated at least three times and their enzymatic activity was estimated using the amount of POA produced in the 1-min reaction divided by the total amount of enzyme in each reaction. This method effectively assessed PZAse activity.

Results

Patient Demographics and MDR Strain Genotyping

A total of 13 of the original 95 clinical isolates (13.7%) were excluded from this study as they were found to be mixed samples, as demonstrated by the presence of more than one band in any of the seven VNTR loci used for MIRU-VNTR genotyping, or they produced an unreadable nesting sequence signal for *pncA*. This left 82 MDR isolates which were then subjected to PZA susceptibility testing and DTM-PCR. There were no significant differences in the age, gender, or treatment history of the patients in the present study (Table 1) who were treated with different doses of PZA. The DTM-PCR indicated that 96% (79/82) of these MDR isolates belonged to the Beijing family of *M. tuberculosis* strains, and there was no significance between the PZA susceptibility profiles of our strains and those representing the Beijing family of MDR isolates (Table 1).

PZA Resistance and pncA Mutation Profiles

The results of the MGIT 960 assays revealed that 28.0% (23/82) of our MDR strains were phenotypically resistant to PZA when evaluated using the standard 100 mg/L concentration and that 15.9% (13/82) were resistant to 300 mg/L PZA. More than half (56.5%, 13/23) of the PZA-resistant MDR strains demonstrated continued PZA resistance even at very high concentrations (300 mg/L).

Our evaluations identified 36 mutation patterns in the *pncA* genes of our MDR clinical strains (Table 2), which occurred across 33 unique mutation sites (nucleotide positions). Of these, 29 were missense mutations, one was a nonsense mutation, three were deletions, two were insertions, and one was a synonymous mutation. A total of 40 strains were found to carry single mutations and only two strains encoded two point mutations each. Interestingly, both double point mutations occurred within the neighboring nucleotide positions of one codon, one was a TG→CC transition at nucleotide positions 104–105, and the other was a CG→GC transition at nucleotides 161 and 162. In addition, we noted a fairly even distribution of these mutations across the coding region of *pncA* with no evident mutational hotspots. Frequency evaluations revealed that the insertion at position 418 (codon 140) of *pncA* had the highest mutation frequency, being identified in four strains, while the other mutations occurred in no more than two individual strains. In addition, we identified a single large-fragment deletion in one strain that resulted in a 435-bp deletion from nucleotide 72 to 506 (Figure S1). Eleven types of *pncA* mutations identified in this study were also reported in the WHO TB GUIDE,[50] and the DST results were congruous except for one mutation types (56 T→C).

Concordance Between pncA Mutation and PZA Resistance

The concordance analyses for each *pncA* mutation and PZA resistance are shown in Table 3. We also noted that molecular evaluations could predict sensitivity to 100 mg/L PZA at a sensitivity of 82.6% (19/23) and a specificity of 61.0% (36/59).

Table 1 Demographic Characteristics of Patients and Genotyping of Clinical Strains Resistant and Susceptible to PZA

	PZA Susceptibility Test		*p* value
	Resistant	Sensitive	
Means of age	42.36	42.71	0.927[c]
Gender[a]			0.335
Male	12	38	
Female	10	16	
Treatment history[b]			0.758
New cases	5	14	
Previously treated	18	42	
Genotyping			1.000
Beijing family	22	57	
Non-Beijing	1	2	

Notes: [a]Six patients without gender documentation. [b]Three patients without treatment history documentation. [c]Calculated by *t*-test.

Table 2 Characteristics of pncA Mutation, PZA Susceptibility and Genotyping in pncA Mutated MDR Strains

Mutation Site (Nucleotide Position)	Nucleotide Change	Amino Acid Position	Amino Acid Replacement	Activity of PZAse	No. of Strains	Pyrazinamide Susceptibility[a]	Beijing/non-Beijing Genotype	Documented in WHO Guide
3	G→A	1	Met→Ile	20.5%	1	Susceptible	Beijing	No
20	T→G	7	Val→Gly	9.79%	1	Resistant[b]	Beijing	Yes, moderate
35	A→C	12	Asp→Ala	0.0%	2	One resistant[b], one susceptible	Beijing	Yes
35	A→G	12	Asp→Gly	0.0%	1	Susceptible	Beijing	Yes, minimal
40	T→C	14	Cys→Arg	14.6%	1	Resistant[b]	Beijing	Yes
56	T→C	19	Leu→Pro	8.0%	1	Susceptible	Beijing	Yes
72–506	435bp fragment deletion	24–169	Frameshift	0.0%	1	Susceptible	Beijing	No
79–92	del CTGGCCC GCGCCAT	27–31	Frameshift	F	1	Resistant[b]	Beijing	No
80	T→G	27	Leu→Arg	0.0%	1	Susceptible	Beijing	No
83	C→A	28	Ala→Asp	21.0%	1	Susceptible	Beijing	No
104–105	TG→CC	35	Leu→Pro	29.0%	1	Resistant	Beijing	No
146	A→C	49	Asp→Ala	0.0%	1	Resistant[b]	Beijing	Yes
161–162	CG→GC	54	Pro→Arg	6.1%	1	Resistant[b]	Beijing	No
170	A→G	57	His→Arg	0.0%	1	Resistant	Beijing	Yes
171	C→A	57	His→Gln	0.0%	1	Resistant	Non-Beijing	Yes
172	T→G	58	Phe→Val	0.0%	1	Susceptible	Beijing	No
195	C→T	65	Synonymous	90.5%	1	Resistant	Beijing	No
203	G→A	68	Trp→Stop	35.2%	1	Susceptible	Beijing	No
203	G→C	68	Trp→Ser	25.3%	1	Susceptible	Beijing	No
203	Ins GT	68	Frameshift	2.0%	1	Susceptible	Beijing	No
212	A→G	71	His→Arg	0.0%	1	Susceptible	Beijing	Yes, minimal
226	A→C	76	Thr→Pro	32.5%	2	One resistant, one susceptible	Beijing	Yes
227	C→T	76	Thr→Ile	0.0%	1	Susceptible	Beijing	No
246	del T	82	Frameshift	0.0%	1	Susceptible	Beijing	No
269	T→C	90	Ile→Thr	17.1%	1	Susceptible	Beijing	No
305	C→T	102	Ala→Val	20.0%	1	Susceptible	Beijing	No
308	A→G	103	Tyr→Cys	12.2%	1	Resistant[b]	Beijing	No
347	T→G	116	Leu→Arg	0.0%	1	Susceptible	Beijing	No
359	T→G	120	Leu→Arg	0.0%	1	Resistant[b]	Beijing	No
398	T→C	133	Ile→Thr	9.8%	1	Resistant	Beijing	Yes, minimal
404	C→A	135	Thr→Asn	12.9%	1	Susceptible	Beijing	No
416	T→C	139	Val→Ala	0.0%	1	Susceptible	Beijing	No
418	Ins TG	140	Frameshift	0.0%	4	Resistant[c]	Beijing	No
463	G→A	155	Val→Met	7.0%	1	Susceptible	Beijing	No
464	T→C	155	Val→Ala	21.8%	1	Susceptible	Beijing	No
467	T→A	156	Leu→Gln	19.9%	1	Susceptible	Beijing	No
478	A→C	160	Thr→Pro	0.0%	1	Resistant[b]	Beijing	No
Total					42			

Notes: [a]Results of pyrazinamide susceptibility testing based on the critical concentration 100 mg/L. [b]Resistant to 300mg/L PZA. [c]All four strains resistant to 100 mg/L PZA and two of them resistant to 300 mg/L PZA. Confidence grading of pncA mutations in WHO GUIDE were classified as high, moderate and minimal.[50]
Abbreviation: F, fail to express the protein.

In addition, of the 42 MDR strains that encoded *pncA* mutations, 19 were shown to be PZA-resistant by the MGIT 960 method, and more than half (54.8%, 23/42) of these *pncA*-mutant MDR strains were shown to be PZA-sensitive. Despite this, the association between *pncA* mutation and PZA resistance was found to be significant (P = 0.001).

Table 3 Concordance of *pncA* Mutation Detection and PZA Susceptibility by MGIT 960

PZA Suscptibility	*pncA* Genotype		Total Strains	*P* value	Sensitivity (%)	Specificity (%)
	Mutant	Wildtype				
100mg/L				0.001	82.6	61.0
Resistant	19	4	23			
Susceptible	23	36	59			
300mg/L				0.009	84.6	55.1
Resistant	11	2	13			
Susceptible	31	38	69			

Evaluations of these strains at 300 mg/L PZA increased the sensitivity of the molecular identification assays to 84.6% (11/13), but the specificity was further reduced to only 55.1% (38/69). In addition, 11 of the MDR strains with *pncA* mutations were shown to be resistant to this increased concentration of PZA when evaluated using the MGIT 960 method, and 73.8% (31/42) of the *pncA*-mutant MDR strains were shown to be PZA-sensitive at 300 mg/L. The association between *pncA* mutation and increased PZA resistance was also significant (P = 0.009). However, it is worth noting that there were two extremely resistant strains that included no *pncA* mutations.

Concordance Between PZAse Enzymatic Activity and PZA Resistance

As previously mentioned, of the 23 phenotypically PZA-resistant strains, 19 were shown to have mutations in their *pncA* gene. We then identified 14 different mutation types in 18 strains that induced a significant decrease in PZAse activity (less than 40%). However, one strain, which encoded a 195 C→T mutation, synonymously maintained 90.5% of its relative PZAse activity. In addition, all nine mutations identified in the 13 highly PZA (300 mg/L)-resistant strains reduced the relative PZAse activity to 20% or less (Figure 1). Moreover, we identified 23 mutations in the *pncA* genes of

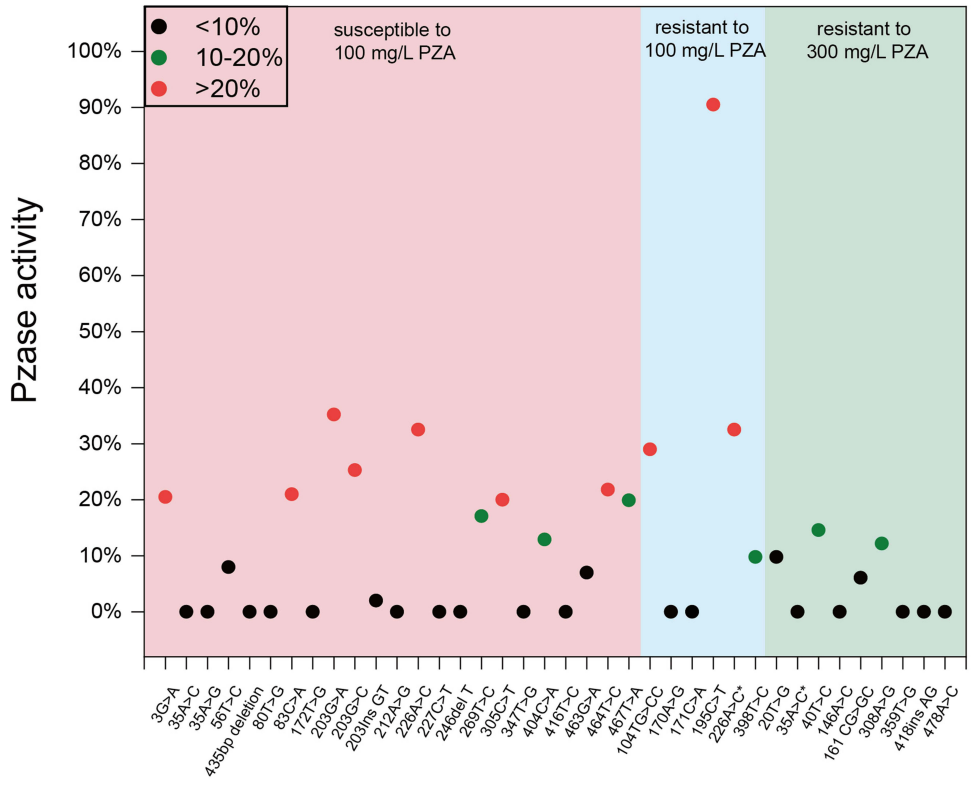

Figure 1 Pyrazinamidase (PZAse) activity of strains. Red: susceptible to pyrazinamide (PZA) at 100 mg/L; Blue: resistant to PZA at 100 mg/L; Green: resistant to PZA at 300 mg/L. *Mutation type with different phenotype.

the 23 phenotypically PZA-susceptible strains, with all of these mutations producing a significant decrease in PZAse activity (< 40%), and 56.5% (13/23) of these mutations even reduced the relative PZAse activity to less than 20%. It is worth noting that we failed to measure the relative enzymatic activity of the "79–92 del 14 bp" deletion owing to its early termination.

Discussion

PZA was first initiated as one of the four first-line drugs for intensive anti-TB treatment in the 1980s, making it a relative newcomer to TB chemotherapy when compared with INH and RIF.[29] However, the recent emergence of both MDR- and extensively drug-resistant-TB has considerably narrowed the range of active therapeutics for the clinical management of TB. One recent study reported that a shorter regimen, in which the injectable agent is replaced by bedaquiline, is highly preferred for patients with resistance to fluoroquinolones.[1] However, the all-oral bedaquiline-containing regimen still includes six agents, and most patients cannot afford bedaquiline because of its high price, which continues to hamper its application. Fortunately, recent research has shown that PZA molecular susceptibility testing could improve the treatment outcomes of MDR-TB without using new drugs in PZA-susceptible patients. This is further supported by the fact that recent results suggest that the current regimen could be shortened to 12 months with comparable success rates to those associated with the WHO-recommended regimen.[9] This emphasizes the importance of PZA in the treatment of drug-resistant TB patients and shows that effective management of MDR-TB is not solely reliant on the development of novel medicines but may also benefit from the application of traditionally effective modalities as determined by DST evaluation.[30–33]

However, unlike the prevalence of other first-line drug resistance, the data around PZA resistance in clinical MDR *M. tuberculosis* strains remain limited. The divergent prevalence of PZA resistance in MDR *M. tuberculosis* clinical strains has been reported in several studies conducted across all six WHO-defined regions.[34–37] A recent systemic review reported a pooled summarized prevalence estimate for PZA resistance of approximately 60.5% (95% CI 52.3–68.6%) in MDR-TB patients and 41.3% (95% CI 29.0–53.7%) in TB patients at high-risk of MDR-TB. Thus, the PZA resistance rate (28.0%) in our study is much lower than this mean value[20] and may be the result of inaccuracies in PZA DST evaluations, or it may simply be a reflection of the less consistent use of PZA for MDR-TB treatment in Henan, China. However, if calculated from the accuracy of the molecular method, excluding the silence mutation, the mutation rate of the samples reached 50%(41/82), which is comparable to the previous studies.

There are many reports describing a wide variety of *pncA* mutations. The majority of these mutations can be classified as missense mutations, with a smaller proportion being described as short insertions or deletions, nonsense mutations in the coding region, or mutations in the putative promoter.[38] All of these classes of mutations were found in our study, and we also identified a relatively rare large-fragment deletion in this gene. Although large-fragment genetic alterations are rarely reported, they often result in truncation.[39] In the present study, we found that the majority of our mutations could be described as single point mutations, resulting in only single amino acid substitutions. We also noted that there were no mutational hotspots in this evaluation.

Given the difficulties in creating an accurate phenotypic PZA susceptibility test, it is only natural that evaluations of the concordance between the phenotypic and genotypic assays for PZA susceptibility have become more common.[40–42] We used a routine critical concentration of PZA (100 mg/L) to complete the first round of sensitivity evaluations and then compared this with the predictive outcomes from our *pncA* mutation assays. These comparisons showed that our mutational evaluations had a concurrence of 82.6%, which is slightly higher than the values reported by studies in Thailand (75%)[45] and Taiwan (80.6%)[48] but is lower than that of the South African (91%) evaluations.[47] The specificity of our study was only 61.0% at the recommended cutoff of 100 mg/L, which is much lower than that of some previous studies that reported specificities of more than 90%.[44,46,47] However, compared to the mutation types documented in the TB GUIDE that were also identified in our study, the results showed a high degree of consistency.

Some of our sequencing and DST results were shown to be slightly contradictory, including the observation that our "large-fragment deletion at 435 bp" mutation, which should induce a code shift and present with PZA resistance, was shown to be susceptible in the DST assays. Similarly, one strain with a T deletion at position 246 and a strain with a GT insertion at position 203 were all shown to be susceptible to PZA. In addition, the DST result of "56 T→C" strain were susceptible,

which was in conflict with that reported in the WHO TB GUIDE too. Conversely, one strain with a "195 C→T" mutation, which induced a synonymous change, recorded a resistant phenotype to PZA.[49] These conflicting results were further verified by evaluating the PZAse activity of these strains, which revealed that the PZAse activity of the "246 del T", "203 in GT", "56 T→C" and "435 bp del" was lower than 10%, while the relative PZAse activity of the "195 C→T" strain remained higher than 90%, which is consistent with synonymous mutation. All of the PZAse activity results were in conflict with the drug sensitivity assays but were consistent with the sequencing results. In addition, there were 23 strains with mutations in *pncA* that all demonstrated a PZA-susceptible phenotype despite an overall reduction in their PZAse activity to less than 40% of the wild-type (WT). In fact, 13 strains reported a relative activity of less than 10% of the WT strain. These results indicate that the phenotypic assays are not necessarily reliable as they suffer from some degree of user inconsistencies.

The inconsistency has been noted in other studies, and some authors have suggested that these discordances might be overcome by using phenotypic evaluations of several critical concentrations of PZA (MICs of ≤64 mg/L, = 128 mg/L, and >128 mg/L) within the BACTEC systems. Werngren et al also suggested that isolates could be classified as susceptible, intermediary, and resistant, respectively.[43] This strategy allows for a potential decrease in both the false resistance and false susceptibility rates by adding evaluations at both lower and higher breakpoints for defining susceptibility and resistance, respectively. Other studies have suggested evaluations at a higher concentration (300 mg/L or 200 mg/L) of PZA as the breakpoint for PZA drug susceptibility.[26,31,48] Thus, we continued to evaluate this higher (300 mg/L) breakpoint and reevaluated our data at this concentration. These evaluations resulted in only a slight increase in the sensitivity of the *pncA* mutation assays (84.6%), but the specificity of these assays was reduced to as low as 55.1%, suggesting that adjusting the critical concentration of PZA is not enough to optimize PZA DST. More detailed experiments are needed to develop a more robust protocol for PZA susceptibility evaluations in MDR-TB strains.

Previous studies have shown that the concordance between the phenotypic and genotypic evaluations of PZA susceptibility in MDR-TB was closely associated with the specific genetic lineage of these strains, such as Beijing/non-Beijing genotypes.[40,46] Dormandy et al reported that all 29 W-Beijing family MDR *M. tuberculosis* strains carrying the same type of nonsynonymous *pncA* mutation showed susceptibility to PZA.[40] One study conducted in Taiwan using 66 MDR *M. tuberculosis* strains produced a higher concordance for *pncA* mutation detection and PZA resistance testing using the MGIT 960 method in non-Beijing strains compared to Beijing strains.[42] In their study, the sensitivity and specificity of their *pncA* mutation detection in Beijing strains were 76.2% and 93.8%, respectively, with both values increasing in non-Beijing strains (86.7% and 100.0%, respectively).[40] These studies suggest that the concordance between *pncA* mutation detection and automatic broth-based PZA resistance detection systems may not be as good in Beijing family MDR *M. tuberculosis* strains as that in non-Beijing family strains. Since 96% of the MDR strains in our study were classified as the Beijing genotype, their genetic lineage might explain the differences in the sensitivity and specificity of our assays when compared to other studies conducted in different places.

Although this study expands our understanding of *pncA* mutations in MDR-TB, there were several limitations. First, all of the MDR strains included in this study were sampled from Henan, China preventing any geographical evaluation. Second, we did not include any upstream *pncA* mutations, which may decrease the sensitivity of the evaluations. Nevertheless, our research may be used to determine the MIC of PZA using a reduced inoculum, via the BACTEC MGIT 960 method, to aid the interpretation of genotypic DST data while minimizing false resistance. Further, we intend to collect more samples from different regions for analysis to overcome the geographic limitations.

Conclusion

Our study illustrates the complexities and challenges associated with PZA susceptibility of *M. tuberculosis* using DST, molecular analysis, and enzyme activity determination. We found that the association between *pncA* mutation and PZA resistance was significant, and the molecular method for detection of PZA resistance is more reliable than the phenotype method. The introduction of the PZAse assay also has a higher consistency with the molecular method. In addition, the BACTEC MGIT 960 automatic method, needs to be established to facilitate both the epidemiological analysis and clinical evaluation of MDR-TB treatment and further our understanding of the relationship between specific mutations and phenotypes.

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Disclosure

The authors report no conflicts of interest in this work.

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