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ORIGINAL RESEARCH

Pyrazinamide Resistance and Mutation Patterns Among Multidrug-Resistant *Mycobacterium tuberculosis* from Henan Province

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Jie Shi
Ruyue Su
Danwei Zheng
Yankun Zhu
Xiaoguang Ma
Shaohua Wang
Hui Li
Dingyong Sun

Henan Province Center for Disease Control and Prevention, Zhengzhou, Henan Province, People's Republic of China

Purpose: This study was designed to identify the phenotypic and genotypic characteristics of pyrazinamide (PZA) resistance among multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) from Henan and to evaluate the efficacy of *pncA, rpsA*, and *panD* mutations in predicting PZA resistance.

Materials and Methods: A total of 152 MDR strains were included in this study. The Bactec MGIT system was used to determine PZA susceptibility for all strains. The *pncA, rpsA*, and *panD* genes were sequenced to identify any mutations, and the sequences were then aligned with the sequence of standard strain H37Rv. Moreover, the correlations between PZA-resistant phenotypes and treatment outcomes were analysed.

Results: Of the152 strains, 105 had a PZA-resistant phenotype, and 102 harboured the *pncA* mutation. The PZA resistance rate was higher in the strains with resistance to all four first-line drugs and those that were pre-extensively drug-resistant (pre-XDR) and extensively drug-resistant (XDR). A total of 100 different *pncA* mutation patterns were identified, including 80 point mutations and 20 insertions/deletions, and 32 new *pncA* mutation patterns were detected. In this study, 13 strains had multiple mutations. Of the11 PZA-resistant strains without *pncA* mutations, two harboured the *rpsA* mutation, and one harboured the *panD* mutation. With PZA susceptibility results as the reference, single-gene *pncA* sequencing had sensitivity of 89.52% and specificity of 89.36%. With the combination of *rpsA* and *panD*, the sensitivity increased to 92.38%, and the specificity remained the same. No significant differences were observed in the sputum smear/culture conversion rate between PZA-resistant patients and PZA-sensitive patients. However, PZA resistance was related to the time to sputum smear/culture conversion ($P = 0.018$).

Conclusion: The combination of *pncA, rpsA*, and *panD* was beneficial for the timely diagnosis of PZA resistance and could provide a laboratory basis for customizing treatment regimens for MDR-TB patients.

Keywords: *Mycobacterium tuberculosis*, multidrug-resistant, pyrazinamide, *pncA*, *rpsA*, *panD*

Introduction

Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB, resistant to at least two of the most powerful first-line anti-TB drugs, isoniazid and rifampin) remains a critical global public health problem due to its high treatment failure rate.[1–3] According to the latest 2019 report from the World Health Organization (WHO), there were approximately10millionnew cases of TB worldwide, and approximately 1.2 million HIV-negative patients died of tuberculosis. China has the second highest MDR-TB burden,

Correspondence: Jie Shi; Dingyong Sun
Tel +86-15803829002; +86-13703865596
Email shijie2280@126.com;
sundy2222@126.com

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after only India, with approximately 120,000 cases of MDR-TB per year. A national survey of drug-resistant tuberculosis in China showed that the incidence of TB is 5.7%, and 25.6% of retreated patients were infected with MDR-TB, significantly higher than global average level.[4] The large number of patients with MDR-TB hinders the effective prevention and control of TB in China.[5,6]

Pyrazinamide (PZA) is an important first-line anti-TB drug, with effective bactericidal activity against both drug-sensitive and MDR strains. As a prodrug, PZA is converted by *pncA*-encoded pyrazinamidase (PZase) into pyrazinoic acid (POA) to exhibit bactericidal activities.[7] The accumulation of POA in cells results in cytoplasmic acidification, which depletes cellular membrane potentials, inhibits various intracellular targets, and eventually leads to cell death.[8] PZA is effective against persistent bacteria in macrophages, which cannot be killed by other anti-TB drugs. Due to its unique antibacterial activity, PZA reduces the treatment time from 12months to6monthswhen it is combined with isoniazid and rifampicin. Studies have shown that72-98%ofPZAresistance is due to *pncA* mutations,[9–11] which are highly diverse and scattered in open reading frames and upstream regulatory regions.[12] In addition to *pncA* mutations, other mechanisms can cause PZA resistance. For example, mutations in *rpsA*, which encodes 30S ribosomal S1 proteins, can alter the POA binding site, causing PZA resistance.[13] Some studies have shown that *panD* is associated with PZA resistance.[11] Researchers are still debating the role of *rpsA* and *panD* mutations in PZA resistance, and more experimental data are needed to clarify the contributions of *rpsA* and *panD* to PZA resistance.

PZA has antibacterial activity only at a low pH; thus, an acidic environment is needed for PZA susceptibility tests. Due to the complex procedures and the failure probability of PZA susceptibility tests, only a few laboratories perform these experiments.[14] However, previous studies have shown that approximately16%of TB patients are PZA resistant. Specifically, the PZA resistance rate is 2–7.5% in non-MDR-TB patients and 36–85% in MDR-TB patients.[15,16] Therefore, the reliable prediction of PZA resistance before treatment facilitates the development of more effective treatments. As the most populous region in China, Henan has the largest number of patients with TB and MDR-TB in the country, making it a hotspot for TB prevention. While PZA is widely used to treat MDR-TB patients, few studies have been conducted to investigate the prevalence of PZA resistance in MDR strains in Henan. In this study, we investigated PZA resistance in MDR-TB patients in Henan and analysed the mutation patterns of PZA resistance-related genes. Moreover, we observed the correlations between PZA resistance and treatment outcomes.

Materials and Methods

Sample Source

MDR-TB patients from 10 regions of Henan during 2018 were included in this study. The patients were followed upfor2years, with monthly sputum smears and *Mycobacterium tuberculosis* cultures during treatment. The study was approved by the Medical Ethics Committee of Henan Provincial Center for Disease Control and Prevention (Number: 2014-KY-012-01). All patients provided informed consent, and this study was conducted in accordance with the Declaration of Helsinki. We collected patient information from medical records. Retreated patients were defined as those receiving more than 1 month of irregular chemotherapy for tuberculosis before presenting at our hospitals.

Drug Susceptibility Testing (DST)

Drug susceptibility testing was performed with WHO-recommended proportion method. The drug concentrations in Lowenstein-Jensenmediumwere 0.2 μg/ml isoniazid (INH), 40 μg/ml rifampicin (RIF), 4μg/ml streptomycin (SM), 2 μg/ml ethambutol (EMB),30 μg/ml kanamycin (KAM), 30 μg/ml amikacin (AMK), 2 μg/ml levofloxacin (OFLX), 1 μg/ml p-aminosalicylic acid (PAS), 40 μg/ml capreomycin (CAM), and 40 μg/ml prothionamide (PTO). Resistant strains were considered when more colonies (>1%) were growing on the drug-containing medium than on the control medium. Bactec MGIT 960 was used for the PZA susceptibility test, and the concentration of PZA in the liquid medium was 100μg/mL. For strains with inconsistent results between DST and DNA sequencing, the tests were repeated, and the minimum inhibitory concentration (MIC) in liquid 7H9 medium was measured. Strains resistant to both isoniazid and rifampicin were defined as MDR strains. In addition to MDR, strains resistant to levofloxacin and at least one second-line injectable anti-tuberculosis drug (amikacin, capreomycin, or kanamycin) were considered extensively drug-resistant (XDR) strains.

DNA Extraction and PCR (Review 1), Sequencing and Characterization of Mutations in *pncA*, *rpsA* and *panD* (Review 2)

Fresh cultured bacteria on Lowenstein-Jensen medium were scraped into500 μL of Tris-EDTA (TE) buffer,

inactivated at 85°C for30min, boiled for 5min, and then centrifuged for later use.[17] Crude DNA extract was used as the template for polymerase chain reaction (PCR). The primers were as follows: *pncA*-5′-AACAGTTCATC CCGGTTC and *pncA*-3′-GCGTCATGGACCCTATATC; *rpsA*-5′-CGGAGCAACCCAACAATA and *rpsA*-5′-GTGG ACAGCAACGACTTC; and *panD*-5′-TCAACGGTTCCG GTCGGCTGCT and *panD*-3′-TATCCGCCACTGCTGC ACGACCTT. The 20μL PCR mixture was prepared as follows: 10 μl of 2 × GoldStar MasterMix (CWBIO Biotech Company, China), 5 μL of DNA template, and 0.2 μM of each primer set. The PCR conditions for amplification were 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. The PCR products were sent to Shanghai Sangon Biotech Company for sequencing. All of the sequence results were aligned with the *pncA, rpsA*, and *panD*genes from reference strain H37Rv (GenBank accession no. NC000962) using DAMAN(version6.0) software.

Genotyping

Spoligotyping analysis was performed in accordance with the previous literature.[18] Briefly, the primers DR3′-CC GAGAGGGGACGGAAAC and DR5′-GGTTTTGGGTC TGACGAC were used for PCR of the extracted genome samples, and then the PCR products were hybridized with a membrane precoated with 43 spacer oligonucleotides to determine the results. The spoligotyping results were aligned through the SITVITWEB website (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). The primersNTF-5′-CCAGATATCGGGTGTGTCGACandNTF-3′-TGCCGT TCTCGAAATCTAAACAA were designed based on the NTF (nuclear transcription factor) region of *M. tuberculosis*.[19] The strain was defined as "modern-type" in the presence of the IS6110 insertion or "ancient type" in the absence of theIS6110 insertion according to a previous report.[20]

Data Analysis

SPSS software, version 19.0, was used for data analysis, and the chi-square test was performed to analyse the correlations between categorical variables.$P<0.05$ was considered statistically significant.

Results

PZA Susceptibility Testing and Patients Demographics

In this study, a total of 152 MDR-TB strains were included, and 105 (69.02%) were resistant to PZA. Moreover, 43 of the152 strains (28.29%) were pre-extensively drug resistant (pre-XDR), 37 (86.04%) were PZA resistant; and 25 (16.44%) were XDR, 23 (92%) were PZA resistant. In addition, 102 (71.71%) strains were resistant to all four first-line anti-tuberculosis drugs, and 82 (80.39%) were resistant to PZA. These results showed that pre-XDR strains, XDR strains, and strains resistant to all four first-line drugs were more likely to develop PZA resistance.

Of the 152 MDR strains, 119 (78.28%) were resistant to streptomycin, 85 (55.92%) were resistant to ethambutol, 35 (23.02%) were resistant to kanamycin, 63 (41.44%) were resistant to levofloxacin, 35 (23.02%) were resistant to amikacin, 25 (16.45%) were resistant to capreomycin, 23 (15.13%) were resistant to prothionamide, and 15 (9.86%) were resistant to p-aminosalicylic acid (Table 1). MDR strains that were resistant to ethambutol, streptomycin, and levofloxacin were more likely to develop PZA resistance.

We further analysed the correlation between PZA resistance and clinical information, including sex, age, close contact history with tuberculosis patients, previous treatment, and tuberculosis genotype. The results showed that the PZA resistance rate was significantly higher among men than women (odds ratio (OR): 2.64, 95% confidence interval (CI): 1.29–5.40, $P= 0.009$), and the percentage of retreated MDR-TB patients in the PZA-resistant group was significantly higher than in the PZA-susceptible group (OR: 4.07,95% CI: 1.92–8.83, $P<0.001$). Analysis of the correlations between the genotypes of these strains and PZA resistance showed that the Beijing family had a higher frequency of PZA resistance than other genotypes and that modern-type strains were more likely to develop PZA resistance than ancient type strains.

Mutations in MDR Strains

The *pncA* genes of 152 strains were sequenced and analysed, and the data showed that the PZA susceptibility results were consistent with the *pncA* mutation for 133 strains. Eight PZA-susceptible strains harboured *pncA* mutations, including three synonymous mutations. Eleven strains were resistant by the test of MGIT960 but harboured

Table 1 PZA Resistance in Different Groups of MDR-TB Patients

Characteristics	No. of Isolates (n=152)	No. of PZA Resistant Isolates (n=105)	No. of PZA Susceptible Isolates (n=47)	OR	95% CI	*P* value
Age						
<20	28	18	10	0.77	0.32–1.85	0.65
21–40	39	26	13	0.86	0.39–1.87	0.69
41–60	61	45	16	1.43	0.71–2.97	0.32
>60	24	16	8	0.88	0.34–2.22	0.81
Sex						
Female	51	28	23	1.00 (reference)		
Male	101	77	24	2.64	1.29–5.40	0.01
Treatment History						
New case	43	20	23	1.00 (reference)		
Retreated	109	85	24	4.07	1.92–8.63	<0.001
Drug resistance to			0			
Ethambutol	85	66	19	2.45	1.23–5.04	0.01
Streptomycin	119	90	29	3.724	1.67–8.31	<0.001
Kanamycin	35	25	10	1.156	0.50–2.65	0.84
Levofloxacin	63	53	10	2.39	1.17–4.89	0.02
Capreomycin	25	17	8	0.942	0.37–2.37	1
P-aminsalicylic acid	15	13	2	3.179	0.69–14.70	0.15
Protionamide	23	16	7	1.027	0.39–2.69	1
Amikacin	35	23	12	0.82	0.37–1.83	0.68
All four first-line drug resistant	109	82	27	2.64	1.26–5.54	0.01
Pre-extensive-drug resistant[a]	53	43	10	2.57	1.15–5.71	0.02
Extensive-drug resistant[b]	27	25	2	7.03	1.59–31.07	<0.001
Spoligotyping						
Beijing family	127	97	30	6.87	2.70–17.50	<0.001
T family[c]	10	6	4	0.652	1.07–2.42	0.49
Other family	14	2 [d]	12 [e]	0.057	0.01–0.27	<0.001
NTF type						
Ancestral type	34	12	22	1.00(reference)		
Modern type	118	93	25	6.82	2.97–15.45	<0.001

Notes: [a]Pre-XDR strains: MDR strains that were also resistant to levofloxacin or any injectable drug (kanamycin, capreomycin, amikacin). [b]XDR strains: MDR strains that were also resistant to levofloxacin and any injectable drug (kanamycin, capreomycin, amikacin). [c]Including the T1, T2, and T3 genotypes. [d]Two strains in the MANU2 family. [e]Seven strains in the MANU2 family, four in the H3 family, and one with an unknown genotype.

no *pncA* mutations. As a result, with PZA susceptibility phenotype as the gold standard, *pncA* mutation detection showed sensitivity of 89.52% (95% CI, 81.64–94.39%) and specificity of 89.36% (95% CI, 76.10–96.01%) in predicting PZA resistance.

Of the 152 MDR strains, 50 harboured no mutations, and102 harboured *pncA* mutations, of which 82 strains had base mutations (including three nonsense mutations and three synonymous mutations), and 20 had frame shift mutations. Moreover, 13 strains harboured multiple mutations, including double mutations in eight strains, triple mutations in three strains, and four mutations in two strains. The remaining 89 strains harboured a single *pncA* mutation, either a single base mutation or a synonymous mutation (Table 2).

Different mutation patterns were detected in these resistant strains, including 14 insertions, 6 deletions, and 80 point mutations. These mutations were scattered

Table 2 Correlation between *pncA* Mutation Patterns and PZAResistance

	No. of PZA Susceptible	No.of PZA Resistant	Total
No mutation	39	11	50
Single mutation	5	81	86
Silent mutation	3	0	3
Multiple mutation	0	13	13
Total	47	105	152

throughout the entire length of the *pncA* gene, including the promoter region. Seven strains harboured a mutation in the promoter region of *pncA*, including an insertion of Gat locus −7 for one strain, a T to C nucleotide substitution at locus −11 for five strains, and an A to G transition at locus −12 for one strain. For the remaining 95 strains, *pncA* mutations were found in the open reading frame. Three strains harboured a large segment deletion, including deletions of codons 79 to 92, codons 189 to 218, and codons 196 to 221. In this study, we detected 32 new mutations that were not found in the GMTV and TBDReaMDB databases or reported in previous studies, including 16 substitution mutations and16 frame shift mutations (Table 3).

The mutations of *pncA* in MDR strains from Henan were diverse. The PZA-resistant strains harboured 100 different mutant types, nine of which were found in multiple strains (Table 3). Specifically, codon 359 was associated with the highest mutation rate, and its mutation (Leuto Arg) was found in eight strains (12.3%). Locus-11 of the *pncA* promoter was associated with the second highest mutation rate; this mutation was found in five strains. Codon 226 mutations (Thr to Pro) were found in four strains (6.8%). In addition, six mutant types were found in two drug-resistant strains.

Eight PZA-susceptible strains harboured *pncA* mutations, including three synonymous mutations. It should be noted that A to T substitution at codon 535 was found in both PZA-resistant and PZA-sensitive strains. PZA susceptibility tests and *pncA* sequencing were repeated for these strains, and the results remained the same. The MICs of PZA were determined with7H9 liquid medium (pH 5.5) for these eight strains. The results showed that the MIC was 12.5μg/mL for three strains with a synonymous mutation, 25 μg/mL for two trains, and 50μg/mL for three strains, which included the strain with a codon535 mutation (Table 4).

Strains without *pncA* mutations could have other mechanisms for PZA resistance. Therefore, other genes associated with PZA resistance were sequenced, including *rpsA* and *panD* (Table 5). No mutations were detected in the promoter regions of *rpsA* and *panD*, and most PZA-resistant strains harbour in g*rpsA* or *panD* mutations were also accompanied by *pncA* mutations. In this study, 72.4% (76/105) of PZA-resistant and 74.5% (35/47) PZA-susceptible clinical strains had eight and one point mutations within their *rpsA* genes, separately. *RpsA* mutations were scattered, although most mutations were located in the180amino acids of the N-terminus and C-terminus. Notably, no *pncA* mutations were detected in PZA-resistant strains harbouring *rpsA* codon357 and codon532 mutations. The CGA to CGC synonymous mutation in *rpsA* codon 212is a non-specific mutation that occurred in both the 68PZA-resistant strains and the 35PZA-susceptible strains. Spoligotyping analysis showed that 96 (93.2%) of the 103 strains with a synonymous *rpsA* mutation belonged to the Beijing family and that two strains belonged to the T family. The *rpsA* nonsynonymous mutation frequencies among PZA-resistant XDR strains and MDR strains were 8% (2/25) and 5.5% (6/109), respectively. The XDR strains had a greater tendency to harbour *rpsA* mutations than the MDR strains. No *panD* mutations were detected in the 47PZA-susceptible strains. Two of the 105PZAdrug-resistant strains harboured *panD* mutations, and one of these strains harboured no *pncA* mutations.

To evaluate the efficiency of DNA sequencing for PZA resistance, we compared the sequencing results and the resistance phenotype (Table 6). With the resistance phenotype as a reference, detection of mutations in *pncA* alone showed sensitivity of 89.52% (95% CI, 81.64–94.39%) and specificity of89.36% (95% CI, 76.10–96.01%); the kappa was 0.76 between the *pncA* mutation and PZA resistance. The concordance between sequencing of *rpsA/panD* and PZA resistance was low, indicating a low diagnostic value for PZA resistance on its own. However, the combination of *rpsA* and *panD* mutations with *pncA* sequencing increased the sensitivity for PZA resistance from 89.52% (95% CI, 81.64–94.39%) to 92.38% (95% CI, 85.54–96.65%) and increased the kappa to 0.80.

Relationship Between Treatment outcome and PZA Susceptibility Phenotype

To investigate the effect of PZA resistance on treatment outcome, we followed up the patients for 24 months to

Table 3 Mutant Profiles of MDR-TB Isolates Within *pncA*

	Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	No. of Isolates
	Substitution				
1	−12	T to C	Promoter	–	5
2	−11	A to G	Promoter	–	1
3	3	ATG to ATA	1	Met to Ile	1
4	11	TTG to TCG	4	Leu to Ser	1
5	14	ATC to AGC	5	Ile to Ser	1
6	19	GTC to TTC	7	Val to Phe	2
7	20	GTC to GGC	7	Val to Gly	1
8	24	GAC to GAA	8	Asp to Glu	1
9	24	GAC to GAG	8	Asp to Glu	1
10	26	GTG to GCG	9	Valto Ala	2
11	26	GTG to GGG	9	Val to Gly	1
12	28	CAG to TAG	10	Gln to Stop	1
13	29	CAG to CCG	10	Gln to Pro	1
14	35	GAC to GCC	12	Asp to Ala	2
15	35	GAC to GGC	12	Asp to Gly	1
16	37*	TTC to GTC	13	Phe to Val	1
17	40*	TGC to GGC	14	Cys to Gly	1
18	40	TGC to CGC	14	Cys to Arg	1
19	41	TGC to TAC	14	Cys to Tyr	1
20	50	GGC to GAC	17	Gly to Asp	1
21	56	CTG to CCG	19	Leu to Pro	1
22	62	GTA to GGA	21	Val to Gly	3
23	71	GGC to GAC	24	Gly to Asp	1
24	77	GCG to GGG	26	Ala to Gly	1
25	83	GCC to GAC	28	Ala to Asp	1
26	94	TTC to GTC	32	Phe to Val	1
27	104	CTG to CCG	35	Leu to Pro	1
28	105*	CTG to CTC	35	Leu to Pro	1
29	123	TAC to TAG	41	Tyr to Stop	1
30	128	CAC to CCC	43	His to Pro	1
31	136	GCA to CCA	46	Ala to Pro	1
32	145	GAC to AAC	49	Asp to Asn	1
33	146	GAC to GGC	49	Asp to Gly	1
34	146	GAC to GCC	49	Asp to Ala	1
35	152	CAC to CGC	51	His to Arg	1
36	160	CCG to TCG	54	Pro to Ser	1
37	161*	CCG to CGG	54	Pro to Arg	1
38	161	CCG to CAG	54	Pro to Glu	1
39	170*	CAC to CGC	57	His to Arg	1
40	171	CAC to CAA	57	His to Gln	1
41	172	TTC to GTC	58	Phe to Val	1
42	185	CCG to CGG	62	Pro to Arg	1
43	194*	TCC to TGC	65	Ser to Cys	1
44	196	TCG to CCG	66	Ser to Pro	1
45	202	TGG to CGG	68	Trp to Arg	1
46	203	TGG to TCG	68	Trp to Ser	1
47	203	TGG to TAG	68	Trp to Stop	2
48	212	CAT to CGT	71	His to Arg	1
49	212*	CAT to CCT	71	His to Pro	1

(*Continued*)

Table 3 (Continued).

	Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	No. of Isolates
50	226	ACT to CCT	76	Thr to Pro	4
51	254	CTG to CGG	85	Leu to Arg	1
52	269*	ATC to ACC	90	Ile to Thr	1
53	287	AAG to AGG	96	Lys to Arg	1
54	290	GGT to GAT	97	Gly to Asp	1
55	305	GCG to GTG	102	Ala to Val	1
56	308	TAC to TGC	103	Tyr to Cys	1
57	314	GGC to GAC	105	Gly to Asp	1
58	317*	TTC to TCC	106	Phe to Ser	1
59	359	CTG to CGG	120	Leu to Arg	7
60	374	GTC to GGC	125	Val to Gly	1
61	374*	GTC to GCC	125	Val to Ala	1
62	398	ATT to ACT	133	Ile to Thr	2
63	407	GAT to GGT	136	Asp to Gly	1
64	412	TGT to CGT	138	Cys to Arg	1
65	416	GTG to GGG	139	Val to Gly	2
66	424	ACG to CCG	142	Thr to Pro	1
67	425	ACG to ATG	142	Thr to Met	1
68	437	GCG to GTG	146	Ala to Val	1
69	463	GTG to ATG	155	Val to Met	1
70	466*	CTG to TTG	154	Leu to Leu	1
71	467*	CTG to CAG	156	Leu to Gln	1
72	467	CTG to CCG	156	Leu to Pro	1
73	478	ACA to CCA	160	Thr to Pro	1
74	511	GCG to ACG	171	Ala to Thr	1
75	511	GCG toCCG	171	Ala to Arg	1
76	519*	GAG to GAA	173	Glu to Glu	1
77	535*	AGC to TGC	179	Ser to Cys	2
78	541*	GAG to TAG	181	Glu to Arg	1
79	543	GAG to GAT	181	Glu to Arg	1
80	555*	AGC to AGT	185	Ser to Ser	1
	deletion				
81	79 to 92*	CTGGCCCGCGCCAT deletion	27	Frameshift	1
82	189 to 218*	CTATTCCTCGTCGTGGCCACCGCATTGCGT deletion	63	Frameshift	1
83	196 to 221*	TCGTCGTGGCCACCGCATTGCGTCAG deletion	64	Frameshift	1
84	200*	C deletion	84	Frameshift	1
85	246*	T deletion	141	Frameshift	1
86	329	A deletion	110	Frameshift	1
	insertion				
87	−7*	G insertion	promoter	–	1
88	121*	ACG insertion	41	Asp insertion	1
89	130*	AG insertion	44	Frameshift	1
90	201*	GT insertion	67	Frameshift	1
91	240*	CGGA insertion	81	Frameshift	1
92	253*	C insertion	85	Frameshift	1
93	256*	G insertion	86	Frameshift	1

(*Continued*)

Table 3 (Continued).

	Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	No. of Isolates
94	268*	T insertion	90	Frameshift	2
95	391	G insertion	131	Frameshift	1
96	392	GG insertion	131	Frameshift	1
97	411*	TG insertion	137	Frameshift	1
98	417*	AG insertion	140	Frameshift	1
99	447*	T insertion	150	Frameshift	1
100	534*	G insertion	179	Frameshift	1
Multiple mutations					
1	11,478, 535,541	TTG to TCG, ACA to CCA, AGC to TGC, GAG to TAG	4,160,179,181	Leu to Ser, Thr to Pro, Ser to Cys, Glu to Arg	1
2	24,35, 254, 412	GAC to GAA, GAC to GCC, CTG to CGG, TGT to CGT	8,12,85,138	Asp to Glu, Asp to Gly, Leu to Arg, Cys to Arg	1
3	105,121, 203	CTG to CTC, ACG insertion, TGG to TCG	35,44	Leu to Pro, Aspinsertion,Trp to Ser	1
4	26,40,398	GTG to GCG, TGC to GGC, ATT to ACT	9,14,133	Val to Ala, Cys to Gly, Ile to Thr	1
5	56,145,463	CTG to CCG, GAC to AAC, GTG to ATG	19,49,155	Leu to Pro, Asp to Asn, Val to Met	1
6	19,437	GTC to TTC, GCG to GTG	71,46	Val to Phe, Ala to Val	1
7	77,171	GCG to GGG, CAC to CAA	26,57	Ala to Gly, His to Gln	1
8	40,104	TGC to CGC, CTG to CCG	14,35	Cys to Arg, Cys to Arg	1
9	37,128,	TTC to GTC, CAC to CCC	13,43	Phe to Val, His to Pro	1
10	160,253	CCG to CTG, C insertion	54,85	Pro to Ser, Frameshift	1
11	196,212	TCG to CCG, CAT to CCT	66,71	Ser to Pro, His to Pro	1
12	29,287	CAG to CCG, AAG to AGG	10,96	Gln to Pro, Lys to Arg	1
13	161,200	CCG to CGG, C deletion	54,84	Pro to Arg, Frameshift	1

Note: *Not found in the TBDRM and GMTV databases or in previous studies.

obtain clinical information. Nine patients failed to provide complete treatment information, including two deaths, two refusals to followup, and five losses. The data showed that after treatment, 93 (93.93%) of the 99 PZA-resistant patients showed sputum smear/culture conversion, while 42 (95.45%) of the 44PZA-susceptible patients showed sputum smear/culture conversion (Table 7). These results indicated no significant difference in sputum conversion rate between PZA-resistant and susceptible patients. However, when the sputum conversion time was segmented and compared with PZA resistance, the results showed that at the end of 6months of treatment, the sputum smear/

Table 4 MIC of PZA-Susceptible Strains Harbouring *pncA* Mutation(s)

Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	No. of Isolates	MIC (μg/mL)
11	TTG to TCG	4	Leu to Ser	1	25
424	ACG to CCG	142	Thr to Pro	1	50
478	ACA to CCA	160	Thr to Pro	1	25
535[a]	AGC to TGC	179	Ser to Cys	1	50
541	GAG to TAG	181	Glu to Arg	1	50
466[b]	CTG to TTG	154	Leu to Leu	1	12.5
519[b]	GAG to GAA	173	Glu to Glu	1	12.5
555[b]	AGC to AGT	185	Ser to Ser	1	12.5

Notes: [a]Present in both PZA-resistant and PZA-susceptible strains. [b]Synonymous mutation.

Table 5 Mutant Profiles of *rpsA* and *panD* among MDR-TB Isolates

	Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	No. of Isolates	*pncA* Nucleotide Change(Amino Acid Change)
PZA resistant isolates						
rpsA						
	93[a]	AAG to AAC	31	Lys to Asn	2	T20G (Val7Gly), A212G (His71Pro)
	357 [a, b]	CTC to CGC	119	Leu to Arg	1	wild-type
	368	GAC to GCC	123	Asp to Ala	1	C171A(His57Gln)
	532[a,b]	AAG to GAG	178	Lys to Glu	1	wild-type
	630[a]	ACC to AGC	210	Thr to Cys	1	G50A(Gly17Asp)
	636[c]	CGA to CGC	212	Arg to Arg	68	
	949[a]	ATC to GTC	317	Ile to Val	1	C161G(Pro54Arg)
	1235	GCC to GTC	412	Ala to Val	1	G145A(Asp49Asn)
panD						
	167	GTC to GCC	56	Val to Ala	1	T40G(Cys14Gly)
	389[b]	GAG to GGG	130	Glu to Gly	1	wild-type
PZA susceptible isolates						
rpsA	636	CGA to CGC	212	Arg to Arg	35	wild-type

Notes: [a]New mutations. [b]No *pncA* mutations. [c]Also present in PZA-susceptible strains.

Table 6 Efficacy Evaluation of Sequencing for PZA Resistance in MDR Strains

gene	PZA Resistant (n=105)		PZA Susceptible (n=47)		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Kappa Coefficient
	Substitutions Mutation and Indel	No Mutation and Synonymous Mutation	Substitutions Mutation and Indel	No Mutation and Synonymous Mutation			
pncA	94	11	5	42	89.52 (81.64–94.39)	89.36 (76.10–96.01)	0.76
rpsA	7	98	0	47	6.67 (2.94–13.68)	100 (92.45–100.00)	0.07
panD	2	103	0	47	1.90 (0.23–6.70)	100 (92.45–100.00)	0.01
pncA/*rpsA*/*panD*	97	8	5	42	92.38 (85.54–96.65)	89.36 (76.10–96.01)	0.8

culture conversion rate was 1.08% in PZA-resistant patients, which was significantly lower than that in PZA-susceptible patients (7.14%) (Table 7), indicating that PZA resistance could affect the time to sputum smear/culture conversion.

Discussion

PZA plays an important role in the treatment of patients with MDR-TB.[21,22] In this study, we investigated PZA resistance in MDR-TB patients in Henan and analysed the correlations between PZA resistance and mutations in PZA resistance-related genes. The results showed that 69% (95% CI, 57.8–71.8%) of MDR strains were PZA resistant, which was similar to that in the Chongqing area (62.4%)[23] and slightly higher than those in previous reports.[24–27] This study showed that retreated MDR-TB patients were more likely to develop PZA resistance (78.0% vs 46.5%), suggesting a correlation between a history of tuberculosis and PZA resistance. The higher PZA resistance rate observed in retreated MDR-TB patients could be related to their previous treatment regimens with PZA. As a result, the role of PZA should be carefully considered in the treatment of MDR-TB patients. It was necessary to perform PZA susceptibility tests to develop an optimal treatment regimen for MDR-TB patients.

Previous reports have revealed a correlation between PZA resistance and fluoroquinolone resistance.[28–30] Similarly, our study showed that PZA resistance was associated with resistance to other anti-tuberculosis drugs,

Table 7 Correlation Between Treatment Outcome and Drug Susceptibility for PZA

Treatment Outcome	PZA Suspustible		PZA Resistant		Chi-Square	p value
	Number of Patients	Percent of Patients (%) (95% *CI*)	Number of Patients	Percent of Patients (%) (95% *CI*)		
Sputum conversion						
Yes	42	95.45 (84.52–99.44)	93	93.93 (87.26–97.74)	0.71	1
No	2	4.54 (0.55–14.57)	6	6.06 (2.26–12.72)		
Sputum conversion time						
6 months after treatment	3	7.14 (1.05–19.48)	1	1.08 (0.03–5.84)	10.01	0.018
12 months after treatment	14	33.33 (19.56–49.55)	20	21.51 (13.65–31.23)		
18 months after treatment	21	50 (34.19–65.81)	45	48.39 (37.89–58.98)		
24 months after treatment	4	9.52 (2.65–22.62)	27	29.03 (20.08–39.36)		

including EMB, SM and OFLX. When exposed to antibiotics such as rifampicin, fluoroquinolones, and aminoglycosides, bacteria will induce the generation of free oxygen radicals in the body and increase the frequency of gene mutations. Given the prolonged use of antituberculosis drugs by MDR-TB patients, we hypothesized that prolonged drug exposure could induce more genetic mutations in *M. tuberculosis*, eventually leading to cross-resistance to PZA and other drugs.

The genotyping results showed that PZA resistance rates were significantly higher in the Beijing family of *M. tuberculosis* than in the non-Beijing family, and PZA resistance rates were higher in the modern subline ages than in the ancient sub line ages. In this study, approximately76.3% of Beijing family and 60% of T family strains harboured PZA resistance, the frequency was significantly higher than those among other genotypes (14.3%). However, due to the small sample size, we were unable to reach any definitive conclusions about correlations with *M. tuberculosis* genotypes, whereas previous studies have shown that PZA resistance was not associated with the genotype.[31]

In this study, the concordance rate was 91.4% between PZA-resistant phenotypes and *pncA* sequences, with discordant results for 13 strains. Eight strains with a *pncA* mutation, including three strains with a synonymous mutation, were susceptible to PZA, while eight strains with wild-type *pncA* were resistant to PZA. To exclude experimental errors, we repeated PZA susceptibility tests for these 13 strains and obtained the same results. The spoligotypes of 16 strains with inconsistent results between resistance and *pncA* gene mutations were analysed, and the results showed that 14 strains belonged to the Beijing genotype and two strains to the T family. We further determined the MICs of PZA-susceptible strains with *pncA* mutations. Except for three strains with a synonymous mutation, the remaining five strains showed low-level PZA resistance (12.5 μg/mL <MIC≤50μg/mL). Drug resistance is the result of the interactions of multiple macromolecules in organisms, including genes, transcripts, and proteins. In addition, some genetic mutations might not necessarily lead to a drug resistance phenotype.[32,33]

The *pncA* mutation rate in PZA-resistant strains varies among different regions, eg, 45.7% in Brazil,[34] 70.6% in Iran,[35] 75.0% in Taiwan,[36] 88% in Chongqing,[23] and 94.1% in Sweden.[37] In this study, 89.52% of the PZA-resistant strains harboured *pncA* mutations. The 102 mutant strains harboured 100 different mutation types (including synonymous mutations) that were scattered throughout the *pncA* gene. Substitution mutations and frame shifts (due to insertion or deletion) were detected in both the open reading frames and promoter regions of *pncA*. In this study, we detected 32 new mutations.[38–42] In addition, 89 of the100mutations were found in only one strain, and the remaining 11 mutations, which contained 35 strains, were shared mutations. Mutations in the promoter region were associated with PZA resistance.[43,44] Seven strains harboured mutations in the promoter region, which down-regulated the *pncA* transcription level, reduced PZ as activity, and ultimately led to PZA resistance. All three strains with a synonymous mutation belonged to the Beijing family. Moreover, out of 35 strains with shared mutations, 28 strains belonged to the Beijing genotype, six strains belonged to the T1 genotype, and one strain belonged to the T2 genotype. Sixteen strains with shared mutations had the same spoligotype and were

genetically clustered. It was found that three of five strains with the promoter substitution mutation (T-12C) and five of seven strains with a substitution mutation at codon 359 were genetically in the Beijing family. Similar genetic classifications were found for substitution mutations at codon 226 (two of four isolates belonging to Beijing family), codon 203 (Beijing family), codon 398 (T family) and codon 416, and all of them were phenotypically resistant. Previous studies have suggested a correlation between *pncA* mutations and gene clustering,[12,45,46] but we were unable to determine gene clustering due to the small sample size and the dominant Beijing genotype.

The11strains without *pncA* mutations could involve other mechanisms of PZA resistance;[11,13,15] therefore, *panD* and *rpsA* mutations were analysed in this study. In 2011, Shi et al reported that *rpsA* could be another PZA resistance-related gene.[13] However, limited data are available to explain the relationship between *rpsA* mutations and PZA susceptibility; thus, the contribution of the *rpsA* mutations conferring PZA resistance has been controversial until now. Alexander et al found no *rpsA* mutations in PZA-resistant isolates but one *rpsA* protein mutation (A364G) in 13 PZA-sensitive strains;[47] Bhuju et al did not find any *rpsA* mutations among either PZA-resistant isolates or PZA-susceptible isolates.[34] In addition, Pang et al found no genetic mutations associated with PZA resistance in the *rpsA* gene among MDR-TB patients.[23] In this study, the*rpsA*mutation frequency (6.7%) in MDR strains was higher than in these reports. A high synonymous mutation frequency was observed at codon212in both PZA-resistant and PZA-susceptible strains. However, no missense or deletion/insertion mutations in *rpsA* were detected in PZA-susceptible strains. Tan et al reported three nonsynonymous mutations in the *rpsA* C-terminal region;[48] Gu et al also identified seven of 26 mutations occurring in the C-terminal region.[24] Moreover, Khan's research found that the majority of *rpsA* mutations were located in nucleotides 973–1051 of the coding region, especially nucleotides 1024–1030.[49] In line with previous reports, we detected a mutation hotspot at the C-terminus of *rpsA*. Moreover, five of the eight *rpsA* mutations have never been described in previous studies, so further research is needed to investigate the effects of these new mutations on PZA resistance. In addition, the XDR strains in this study had a greater tendency to harbour *rpsA* mutations than the MDR strains, also demonstrating that *rpsA* mutations might be related to PZA resistance. While previous studies have shown that *panD* is a target for PZA resistance in *M. tuberculosis*, few studies have been conducted to investigate *panD* mutations in clinical strains. This study showed that two (1.9%)PZA-resistant strains harboured a *panD* mutation, one of which had wild-type *pncA*. In summary, we identified eight PZA-resistant strains without any known mutations, indicating that additional unknown mechanisms are involved in PZA resistance and requiring further research. For PZA resistance, the combination of all three genes sequenced achieved sensitivity of 92.38% and specificity of 89.36%, consistent with other reports. Considering the insufficient reliability and inconsistency between phenotypes and the drug susceptibility test, the sequencing of all three PZA-resistant genes could evaluate the resistance of PZA more effectively.

Finally, we analysed the outcomes of 124 patients under different treatment regimes. The sputum smear/culture conversion rate was slightly higher in PZA-sensitive patients than in PZA-resistant patients (95.5%vs93.9%), but the difference was not statistically significant. However, subgroup analysis showed that at the end of 6 months of treatment, the sputum smear/culture conversion rate was significantly higher in PZA-sensitive MDR-TB patients than in PZA-resistant patients (7.14%vs1.08%), suggesting that PZA resistance prolonged the time to sputum smear/culture conversion.

This study had several limitations. First, the sample size was small, and all of the strains were derived from our region. As a result, the results might not be representative and might not apply to other regions; therefore, more samples are needed in the future. Second, we detected 32 new *pncA* mutations and five new *rpsA* mutations, and further research is needed to investigate their effects on PZA resistance.

In summary, this study was the first to investigate the molecular characterization ofPZA resistance in MDR strains from Henan. The results showed that PZA resistance was substantially related to *pncA* mutations, which were scattered and diverse. In addition, *rpsA* and *panD* mutations were related to PZA resistance only in MDR strains without *pncA* mutations. With the resistance phenotype as the reference, molecular diagnosis with the combination of *pncA, rpsA*, and *panD* achieved sensitivity of98.1% and specificity of92.3%. Given the high PZA resistance rate in MDR-TB patients and that PZA resistance prolonged the time to sputum smear/culture conversion, the effects of PZA should be considered carefully for MDR-TB patients in high TB burden regions. In

conclusion, the combination of *pncA, rpsA*, and *panD* enabled rapid and accurate prediction of PZA resistance, helped guide the use of PZA and optimized treatment therapy for MDR-TB patients in Henan.

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Disclosure

The authors report no conflicts of interest in this work.

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