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Prevalence and transmission of pyrazinamide resistant *Mycobacterium tuberculosis* in China

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SUMMARY

Pyrazinamide (PZA) is an important first-line anti-tuberculosis drug, however, there are relatively few available data on PZA resistant (PZA-R) rate in China. From June 2009 to June 2012, we selected 493 isolates from five field settings in China to investigate PZA-R by *pncA* gene sequencing. The result showed that PZA-R rate was 1.0% (2/196) among pan-susceptible isolates, 3.1% (4/130) among isoniazid (INH) mono-resistant isolates, 14.0% (6/43) among rifampin (RIF) mono-resistant isolates and 43.5% (54/124) among multidrug resistant (MDR) isolates. MDR tuberculosis (TB), RIF mono-resistance, and retreatment were found to be risk factors for PZA-R. Newly diagnosed PZA-R TB patients and clustered isolates with identical *pncA* mutations indicate that transmission of PZA-R isolates plays an important role in emergence of PZA-R TB. The results suggest that, it is necessary to conduct PZA susceptibility test among MDR isolates and modify the treatment regimens accordingly.

Keywords

Mycobacterium tuberculosis; Pyrazinamide resistance; Transmission

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Competing interests
None declared.

Ethical approval
The Ethics Committees of the Institutes of Biomedical Sciences in Fudan University and the Shanghai Municipal Center for Disease Control and Prevention, Shanghai, approved the protocol for this study.

1. Introduction

Pyrazinamide (PZA) is a first-line drug and an important component used for the treatment of both drug-susceptible and drug-resistant tuberculosis (TB). Combined with other anti-TB drugs, PZA shows a remarkable therapeutic effect [1,2], because of its unique sterilizing activity to kill semi-dormant or persistent *Mycobacterium tuberculosis* [3]. Specifically, in multidrug resistant (MDR) TB therapy, it has a strong effect on the success rates of treatment [4]. So, the absence of PZA in the regimens would lead to poor treatment outcome [5–7].

At present, there are two main methods for drug susceptibility testing (DST) of *M. tuberculosis*: the phenotypic DST by traditional culture, and the molecular drug susceptibility test (mDST) by detecting drug resistant mutations. However, both phenotypic DST and mDST have limitations to detect PZA-R. The accuracy and reproducibility of PZA phenotypic DST are not satisfactory [8,9], because it must be conducted under acidic condition (pH 5.5–6). The growth of *M. tuberculosis* is inhibited at such a low pH and many factors such as inoculum size could lead to inaccurate results. For mDST, because the molecular mechanism of PZA-R is not completely understood, the PZA mDST based on detecting *pncA* mutations could provide only about 90% specificity and sensitivity [10]. According to studies conducted in different areas of China, the specificities of *pncA* sequencing to predict PZA-R were more than 90% [11–13]. Therefore, for large scale and multi-center PZA-R investigation in China, mDST is a more reliable and objective method.

China is one of the high drug resistant TB burden countries, but there are relatively few available data on PZA-R rates. Studies showed that PZA-R rates were 35.6% among drug resistant TB in Hong Kong [14] and 43.1% among MDR-TB in Zhejiang [13]. However these studies were based on samples collected from single hospital, which could not reflect the real prevalence of PZA-R in the general population. In this study, we used the *pncA* mutations to predict PZA-R among *M. tuberculosis* clinical isolates collected from a population based epidemiological study and estimated the prevalence and risk factors of PZA-R in China.

2. Materials and methods

2.1. Study population and isolates

In a previous study, we collected all *M. tuberculosis* isolates from five counties in different provinces of China (Songjiang in Shanghai, Wusheng in Sichuan, Pingguo in Guangxi, Wuchang in Heilongjiang, and Weishi in Henan provinces respectively) between June 2009 and June 2012 [15]. The data of patient information and isolates genotype (Beijing genotype and variable number tandem repeat (VNTR) genotype) was described in our previous study [15]. The isolates were considered to be clusters only when they shared the same VNTR genotype within each field setting. We included all drug resistant isolates (resistance to rifampin (RIF) and/or isoniazid (INH)), and randomly selected susceptible isolates (sensitivity to both RIF and INH) collected during the same period as susceptible group for comparison.

2.2. Drug susceptibility test

The isolates cultured directly from sputum were used to do phenotypic DSTand mDST. The phenotypic DST was conducted using the proportion method on Lowenstein–Jensen medium [16], and the DNA used in mDST was extracted by boiled lysis method [17]. Both phenotypic DST and mDST were performed to detect INH and RIF susceptibilityof the isolates. The mDSTof INH and RIF was performed using multiplex real-time PCR melting curve assay [18]. The isolates with consistent results of both methods were included in this study. The fluoroquinolones (FQs) and PZA susceptibility were determined by mDST using DNA sequencing. FQs mDST was performed on all MDR isolates by sequencing quinolone resistance-determining region (QRDR) of the *gyrA* gene [19]. PZA-R was predicted by detecting mutations among the whole *pncA* gene and its upstream region. We used two pairs of primers (pncA-721-F: GCTGGTCATGTTCGCGATCG, pncA-721-R: CGCTTGCGGCGAGCGCTCCA and pncA-951-F: CTGTCACCGGACGGATTTG, pncA-951-R: ATCGCGATGGAACGTGATA) to amplify and sequence 721 bp and 951 bp fragments. The pncA-951 primers were used only under the condition that the pncA-721 primers were failed to amplify the target fragment. All the mutations within the *pncA* gene and its upstream region were confirmed by both-direction sequencing.

According to the results of INH and RIF susceptibility, the isolates were classified into four categories: pan-susceptible (RIF and INH susceptible), INH mono-resistant, RIF mono-resistant and MDR (both RIF and INH resistant). In MDR group, the FQs resistant isolates were defined as pre-XDR. The isolates with non-synonymous mutations in *pncA* gene and its upstream region were considered as PZA-R.

2.3. Statistical analyses

Data were analyzed by using Stata software (version 13.1/SE, Stata Corp, College Station, Texas). We used univariate and multivariable logistic regression modeling to calculate odds ratios (OR) and adjusted odds ratio (aOR) for factors associated with PZA-R. We used a forward, stepwise approach to select the multivariable model. Factors with biological plausibility and P value < 0.2 in the univariate analysis were considered in the final model. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the patients and isolates

During the period from June 2009 to June 2012, 2274 culture-positive TB cases were diagnosed in the five settings [15]. With the exception of 60 isolates without DST data and 39 isolates with inconsistent results between phenotypic DST and mDST, there were 130 INH mono-resistant, 43 RIF mono-resistant and 124 MDR isolates (including 35 pre-XDR isolates). To estimate the proportion of PZA-R, we included all 297 drug-resistant isolates and randomly selected 196 isolates from 1878 pan-susceptible isolates. There were no statistically differences between selected and unselected pan-susceptible cases with regards to the characteristics of patients such as gender, age and TB treatment history. Thus, finally 493 isolates were included in this study, of which, 75.3% patients were male, the median age

was 42 (interquartile range, 28–57), 78.3% were newly diagnosed cases and 77.5% isolates belonged to Beijing genotype strain.

3.2. Mutations types and prevalence of PZA resistance

To detect PZA-R related gene mutations, DNA sequencing of the *pncA* gene and its upstream region was performed on all isolates. In total, 66 out of the 493 isolates had non-synonymous mutations. Except of one isolate with two mutations (Y103STOP and D126G), the remaining 65 isolates carried a single mutation. Forty-eight mutation types were detected among the 66 isolates, including six insertion mutations (five frame shift mutations) and 42 point mutations (three mutations in the upstream region) (Table 1). No synonymous mutation was detected in all isolates.

The 66 isolates harboring non-synonymous mutations in the *pncA* gene and its upstream region were defined as PZA-R. Thus, the PZA-R rate was 1.0% (2/196) among pan-susceptible isolates, 3.1% (4/130) among INH mono-resistant isolates, 14.0% (6/43) among RIF mono-resistant isolates, 43.5% (54/124) among MDR and 54.3% (19/35) among pre-XDR isolates (Table 2).

3.3. Risk factors of PZA resistance

In order to identify the risk factors for PZA-R, we performed univariate analysis and multivariable logistic regression analysis. Table 3 shows results of univariate analysis. Resistance of PZA significantly increased as the isolate being resistant to more anti-TB drugs (from mono-resistance to pre-XDR, P value < 0.001 for trend). In the multivariable regression analysis, retreatment (adjusted odds ratio [aOR], 2.36; 95% confidence interval [CI], 1.21–4.67, P value < 0.05), RIF mono-resistance (aOR, 7.43; 95% CI, 1.27–43.37, P value < 0.05), MDR (aOR, 45.05; 95% CI, 10.32–196.62, P value < 0.001) and pre-XDR (aOR, 86.36; 95% CI, 18.17–410.35, P value < 0.001) were independently associated with PZA resistance. These variables remained significantly associated with PZA resistance after adjustment for sex, age and Beijing genotype.

3.4. Transmission of PZA-R isolates

The emergence of drug resistance could be due to acquired drug resistance caused during treatment, or primary drug resistance caused by transmission of drug resistant isolates. Drug resistance in newly diagnosed TB patients is generally considered as primary drug resistance, while drug resistant TB patients with clustered isolates are suggestive of recent transmission. Among the 66 PZA-R cases, 48.5% (32/66) were newly diagnosed TB patients, indicating the occurrence of primary drug resistance. In order to identify potential recent transmission of PZA-R isolates during the study period, we analyzed the VNTR data of all 493 isolates. Results showed that PZA-R isolates were involved in 11 clusters, in which 7 clusters had at least 2 isolates with identical *pncA* mutations (Figure 1). Clustered isolates shared the identical *pncA* mutation, suggesting recent transmission of PZA-R isolates. Patients' information showed that five PZA-R patients in cluster 2 and two patients in cluster 3 lived in the same village, thus providing probable epidemiological links for the recent transmission of PZA-R *M. tuberculosis*.

4. Discussion

This study covered five settings located in different provinces of China. We selected all drug resistant and randomly selected drug susceptible *M. tuberculosis* isolates, to predict PZA-R prevalence by mDST. The results showed that 81.8% of PZA-R isolates were MDR-TB isolates; and that MDR, pre-XDR, RIF mono-resistance and retreatment were risk factors for PZA-R. Furthermore, we observed that 48.5% of the PZA-R was primary drug resistance. Clustered isolates with identical *pncA* mutations were also detected, suggesting recent transmission of PZA-R isolates.

PZA is an important anti TB drug, both the WHO and the China CDC recommend PZA as a key component for treating new and re-treated patients [20]. However, PZA-R isolates can lead to serious negative effects on TB treatment [4]. Therefore, it is necessary to modify the treatment regimens of patients with PZA-R isolates. Our data showed the PZA-R rate among MDR isolates was as high as 43.5%. Hence, for MDR-TB, it is of importance to perform PZA susceptibility testing before prescribing the treatment regimens.

In this study, nearly half of the PZA-R cases were new TB patients, in the United States, the national PZA-R tuberculosis survey also found that up to 76.5% of the PZA-R MDR strains are new TB patients [21]. These PZA-R cases were regarded as primary drug resistance caused by remote or recent transmission of PZA-R strains. Our data showed that 27.3% (18/66) PZA-R isolates shared the identical *pncA* mutation and VNTR genotype, indicating recent transmission. For example, in Henan field site, the cluster rate of PZA-R isolates was as high as 52.2% (12/23). However, the study of MDR strains in Russia showed that identical *pncA* mutations existed in smaller clusters, suggesting a weak transmissibility of PZA-R isolates [22]. Whether *pncA* mutations induce a fitness cost that impairs transmission still need further study.

Most (81.2%, 39/48) of *pncA* mutation types identified in this study are associated with a high confidence of PZA resistance according to at least one TB drug resistance databases [23,24] or have been reported in PZA resistant strains [11–13,25–32] (Table 1), suggesting a strong correlation with PZA phenotypic resistance. But the mDST of PZA has an inevitable number of false positive and false negative results, there may be about 10% undetected and wrong defined PZA-R isolates by this method [10].

5. Conclusion

By detecting *pncA* mutation of *M. tuberculosis* isolates from five provinces of China, we have shown that the PZA-R rate was as high as 43.5% in MDR isolates. Our results also showed that MDR, pre-XDR, RIF mono-resistance and retreatment were risk factors for PZA-R. Finally, since transmission of PZA-R isolates play important role in emergence of PZA-R TB, it is necessary to regularly conduct PZA susceptibility testing among MDR-TB patient and modify the treatment regimens accordingly.

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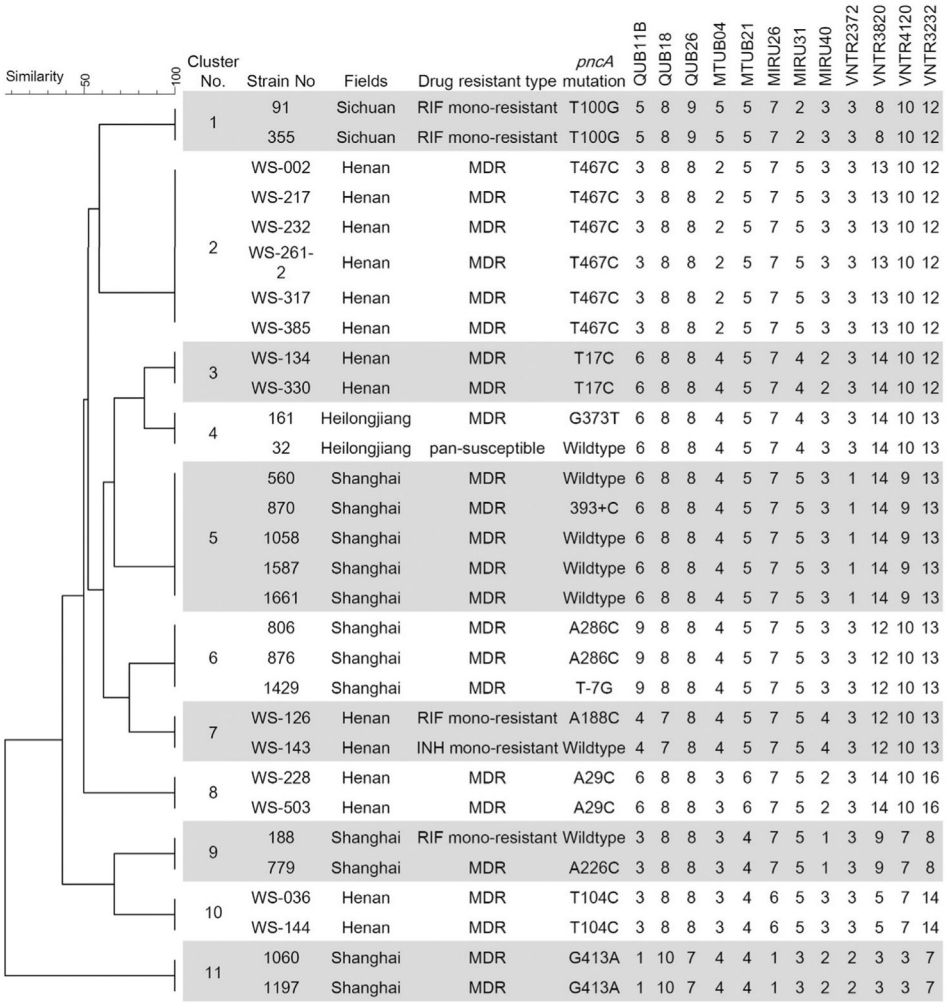

Cluster No.	Strain No	Fields	Drug resistant type	*pncA* mutation	QUB11B	QUB18	QUB26	MTUB04	MTUB21	MIRU26	MIRU31	MIRU40	VNTR2372	VNTR3820	VNTR4120	VNTR3232
1	91	Sichuan	RIF mono-resistant	T100G	5	8	9	5	5	7	2	3	3	8	10	12
	355	Sichuan	RIF mono-resistant	T100G	5	8	9	5	5	7	2	3	3	8	10	12
2	WS-002	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
	WS-217	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
	WS-232	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
	WS-261-2	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
	WS-317	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
	WS-385	Henan	MDR	T467C	3	8	8	2	5	7	5	3	3	13	10	12
3	WS-134	Henan	MDR	T17C	6	8	8	4	5	7	4	2	3	14	10	12
	WS-330	Henan	MDR	T17C	6	8	8	4	5	7	4	2	3	14	10	12
4	161	Heilongjiang	MDR	G373T	6	8	8	4	5	7	4	3	3	14	10	13
	32	Heilongjiang	pan-susceptible	Wildtype	6	8	8	4	5	7	4	3	3	14	10	13
5	560	Shanghai	MDR	Wildtype	6	8	8	4	5	7	5	3	1	14	9	13
	870	Shanghai	MDR	393+C	6	8	8	4	5	7	5	3	1	14	9	13
	1058	Shanghai	MDR	Wildtype	6	8	8	4	5	7	5	3	1	14	9	13
	1587	Shanghai	MDR	Wildtype	6	8	8	4	5	7	5	3	1	14	9	13
	1661	Shanghai	MDR	Wildtype	6	8	8	4	5	7	5	3	1	14	9	13
6	806	Shanghai	MDR	A286C	9	8	8	4	5	7	5	3	3	12	10	13
	876	Shanghai	MDR	A286C	9	8	8	4	5	7	5	3	3	12	10	13
	1429	Shanghai	MDR	T-7G	9	8	8	4	5	7	5	3	3	12	10	13
7	WS-126	Henan	RIF mono-resistant	A188C	4	7	8	4	5	7	5	4	3	12	10	13
	WS-143	Henan	INH mono-resistant	Wildtype	4	7	8	4	5	7	5	4	3	12	10	13
8	WS-228	Henan	MDR	A29C	6	8	8	3	6	7	5	2	3	14	10	16
	WS-503	Henan	MDR	A29C	6	8	8	3	6	7	5	2	3	14	10	16
9	188	Shanghai	RIF mono-resistant	Wildtype	3	8	8	3	4	7	5	1	3	9	7	8
	779	Shanghai	MDR	A226C	3	8	8	3	4	7	5	1	3	9	7	8
10	WS-036	Henan	MDR	T104C	3	8	8	3	4	6	5	3	3	5	7	14
	WS-144	Henan	MDR	T104C	3	8	8	3	4	6	5	3	3	5	7	14
11	1060	Shanghai	MDR	G413A	1	10	7	4	4	1	3	2	2	3	3	7
	1197	Shanghai	MDR	G413A	1	10	7	4	4	1	3	2	2	3	3	7

Figure 1.

The clustered isolates with *pncA* mutations. 12 loci VNTR based dendrogram and isolates profiles of 11 clusters in which *pncA* mutant isolates were involved. Abbreviations: INH, isoniazid; RIF, rifampin; MDR, multidrug resistant.

Table 1

Mutations in the *pncA* gene and its upstream region.

Mutation type	Amino acid change	No. of isolates	Relationship to PZA phenotypic resistance			
			Miotto et al.*	TB-DreamDB[y]	MUBII-TB-DB[z]	Reported elsewhere [ref.][§]
A-11C	N.	1	Very high confidence	Reported	Reported	
A-11G	N.	3	Very high confidence	High confidence	High confidence	
T-7G	N.	1	Very high confidence	Unreported	Unreported	
T2C	M1T	1	Very high confidence	Reported	Reported	
T17C	I6T	2	Very high confidence	Unreported	Unreported	
G19T	V7F	1	Very high confidence	Reported	Reported	
A29C	Q10P	3	Very high confidence	High confidence	Reported	
T40G	C14G	1	Unreported	Unreported	Unreported	
G50T	G17V	1	Unreported	Reported	High confidence	
T100G	Y34D	2	Unreported	Unreported	Unreported	[26]
C102A	Y34STOP	1	Very high confidence	Unreported	Unreported	
T104C	L35P	2	Very high confidence	High confidence	Unreported	
C123A	Y41STOP	1	Unreported	Reported	Reported	
A139G	T47A	1	Not involved in resistance	High confidence	Reported	[12,13,27]
A146C	D49A	1	Very high confidence	High confidence	Reported	
A152G	H51R	1	Very high confidence	High confidence	High confidence	
166 + G	56Frameshift	1	Unreported	Unreported	Unreported	
C174G	F58L	1	Very high confidence	Reported	Reported	
T175C	S59P	2	Unreported	Reported	Reported	[26,28]
186 + GGAC	62insDY	1	Unreported	Unreported	Unreported	
TATTCCTC GTCGTG	SSSW					
A188C	D63A	1	Unclear role	Unreported	Unreported	[27]
T192G	Y64STOP	1	Very high confidence	Unreported	Unreported	
C211T	H71Y	1	Very high confidence	Reported	Reported	
A226C	T76P	3	Very high confidence	High confidence	Reported	
T283G	Y95D	1	Unreported	Unreported	Unreported	
A286G	K96E	1	Very high confidence	Reported	Reported	

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Mutation type	Amino acid change	No. of isolates	Relationship to PZA phenotypic resistance			
			Miotto et al.*	TB-DreamDB†	MUBII-TB-DB‡	Reported elsewhere [ref.]§
A286C	K96Q	2	Very high confidence	Reported	Reported	
T307G	Y103D	1	Very high confidence	Unreported	Unreported	
C309G	Y103STOP	1¶	Very high confidence	High confidence	Reported	
C312A	S104R	1	Very high confidence	Unreported	Unreported	
G373T	V125F	1	Very high confidence	Unreported	Unreported	
A377G	D126G	1¶	Unreported	Unreported	Unreported	
390 + G	130Frameshift	1	Very high confidence	Unreported	Unreported	
390 + GG	130Frameshift	1	Very high confidence	Unreported	Unreported	
393 + C	131Frameshift	1	Unreported	Unreported	Unreported	
A403C	T135P	1	Very high confidence	Reported	High confidence	
A407C	D136A	1	Unreported	Unreported	Unreported	
G413A	C138Y	2	Unreported	High confidence	Reported	[29,30]
T416G	V139G	2	Very high confidence	High confidence	High confidence	
G436A	A146T	1	Unreported	Reported	Reported	[11,13,28,31]
C442T	R148C	1	Unreported	Unreported	Unreported	
A460G	R154G	1	Unclear role	Reported	Reported	[22,32]
G463A	V155M	1	Very high confidence	Unreported	Unreported	
T467C	L156P	7	High confidence	Unreported	Unreported	
G484A	G162S	1	Unreported	Unreported	Unreported	[11,29]
T490C	S164P	1	Very high confidence	Unreported	Unreported	
514 + C	172Frameshift	1	Unreported	Unreported	Unreported	
A523G	M175V	1	Very high confidence	Reported	Reported	

*A Multicenter Study with 1950 clinical isolates [25].

†TB Drug Resistance Mutation Database (TB-DreamDB) (www.tbdreamdb.com) [24].

‡MUBII-TB-DB (https://umr5558-bibiserv.univ-lyon1.fr/mubii/mubii-select.cgi) [23].

§Previously reported in PZA-R isolates.

¶The two mutations (Y103STOP and D126G) coexisted in one strain.

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Table 2

PZA resistance among 493 *M. tuberculosis* isolates.

PZA-R	Pan-susceptible		INH mono-resistant		RIF mono-resistant		MDR (including pre-XDR)		Pre-XDR	
Guangxi	2/30	6.7%	0/19	0%	2/11	18.2%	4/13	30.8%	0/0	N.
Heilongjiang	0/21	0%	1/15	6.7%	0/8	0%	4/14	28.6%	0/2	0%
Henan	0/42	0%	1/28	3.6%	1/4	25.0%	21/28	75.0%	8/8	100.0%
Sichuan	0/28	0%	0/20	0%	3/11	27.3%	10/30	33.3%	2/7	28.6%
Shanghai	0/75	0%	2/48	4.2%	0/9	0%	15/39	38.5%	9/18	50.0%
Total	2/196	1.0%	4/130	3.1%	6/43	14.0%	54/124	43.5%	19/35	54.3%

Abbreviations: PZA, pyrazinamide; INH, isoniazid; RIF, rifampin; MDR, multidrug resistant.

Table 3

Univariate analysis of risk factors of PZA resistant tuberculosis.

Characteristic	PZA-S (n = 427) No. (%)	PZA-R (n = 66) No. (%)	PZA-R VS PZA-S	
			OR (95% CI)	*P* value
Sex				
Male	319 (86.0)	52 (14.0)	1	
Female	108 (88.5)	14 (11.5)	0.80 (0.40–1.53)	0.47
Age				
< 35	146 (86.4)	23 (13.6)	1	
35–55	156 (84.3)	29 (15.7)	1.18 (0.63–2.24)	0.58
>55	120 (90.9)	12 (9.1)	0.63 (0.28–1.40)	0.22
Unknown	5 (71.4)	2 (28.6)	2.54 (0.23–16.56)	0.27
TB history				
New	354 (91.7)	32 (8.3)	1	
Re-treated	58 (65.9)	30 (34.1)	5.72 (3.09–10.50)	<0.001
Unknown	15 (78.9)	4 (21.1)	2.95 (0.67–9.99)	0.06
Beijing strain				
No	103 (92.0)	9 (8.0)	1	
Yes	324 (85.0)	57 (15.0)	2.01 (0.95–4.78)	0.06
Drug resistance profile				<0.001 *
Pan-susceptibility	194 (99.0)	2 (1.0)	1	
INH mono-resistance	126 (96.9)	4 (3.1)	3.08 (0.55–17.12)	0.18
RIF mono-resistance	37 (86.0)	6 (14.0)	15.73 (2.85–86.8)	<0.001
MDR	54 (60.7)	35 (39.3)	62.9 (11.3–350.5)	<0.001
Pre-XDR	16 (45.7)	19 (54.3)	115.2 (14.5–912.6)	<0.001
FQs resistance in MDR (n = 124)				
No	54 (60.7)	35 (39.3)	1	
Yes	16 (45.7)	19 (54.3)	1.83 (0.77–4.36)	0.13

Abbreviations: PZA, pyrazinamide; INH, isoniazid; RIF, rifampin; MDR, multidrug resistant.

* *P* value for trend of chi-square test.