

1 ***Title page:***

2 Emergence and genetic characteristics of pyrazinamide resistant
3 tuberculosis in China, a multi-center study

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20 The running title: PZA resistance in China

ABSTRACT

Background: The aim of this study was to investigate the rates of pyrazinamide (PZA) resistance and the associated risk factors as well as to evaluate the *pncA* gene loci as a marker for PZA resistance in China.

Method: A population-based multi-center study of pulmonary TB cases was carried out from 2011 to 2013 in four Chinese district/counties with different geographic and socio-economic features. Testing for multidrug-resistant tuberculosis (MDR-TB) and susceptibility to PZA were determined by the proportion method on Löwenstein-Jensen medium and BACTEC MGIT 960 respectively. Mutations in the *pncA* gene were identified by sequencing.

Results: Among 878 culture positive cases, 147(16.7%) were resistant to PZA, with a significantly higher proportion among MDR isolates compared to the drug sensitive isolates (30.2% VS 7.7%, $p<0.001$). Totally, 136 isolates had a non-synonymous *pncA* mutation, with a comparable diagnostic performance between Beijing family and non-Beijing family as well as between MDR-TB and 1st-line drug susceptible TB. Furthermore, the mutations in isolates with high-level PZA resistance (MIC>500mg/L) were observed mainly in three regions of the *pncA* gene (codon 51-76, codon 130-142 and codon 163-180). Patients with prior treatment history had a significantly higher risk for PZA mono-resistance (OR:2.86,95%CI:1.363-6.015) and MDR PZA resistance (OR:6.47, 95%CI:3.186-13.15), while the additional factors associated with MDR-PZA resistance were the patient's age (OR:1.02, 95%CI:1.003-1.042), lung cavity (OR:2.64, 95%CI:1.296-5.391).

43 *Conclusions:* These findings suggest that it is a priority to identify PZA resistance in
44 MDR-TB and a rapid molecular diagnostic test based on *pncA* mutations in the
45 Chinese settings where MDR-TB prevalence is high should be developed.

46

47 *Keywords:*

48 *Mycobacterium tuberculosis*; China; Pyrazinamide resistance; *pncA* gene; Minimum
49 inhibitory concentration; Sensitivity and specificity

50 *Text*

51 **BACKGROUND**

52 Drug-resistant *M. tuberculosis* (Mtb), which predominantly arises from the selection of
53 naturally resistant bacterial strains through pressure from inappropriate chemotherapy,
54 poses a serious challenge to global TB control (1). Because of its high bactericidal
55 effect in an acidic environment, pyrazinamide (PZA) is usually recommended for the
56 treatment of PZA susceptible MDR-TB throughout the entire duration of the regimen
57 (2). An increasing number of PZA resistant (R) strains has however been observed (1)
58 and without precise knowledge of PZA susceptibility, its unstandardized utilization in
59 treatment regimen could lead to resistance against other antibiotics as well (particularly
60 against efficient oral drugs, such as fluoroquinolones (FQ), that are initially included in
61 the regimen). The unavailability of low cost drug susceptibility testing (DST)
62 techniques, in addition to safety considerations related to the handling of MDR-TB
63 strains, further inhibits our understanding of the scale of PZA resistance in
64 resource-limited areas.

65

66 PZA is a pro-drug that needs to be converted to an active form, pyrazinoic acid, by the
67 bacterial pyrazinamidase (PZase) enzyme which is encoded by *pncA*.
68 mutations/variations in *pncA* leading to the loss of PZase activity is the major
69 mechanism leading to PZA-resistance (PZA-R)(3). However, while high numbers of
70 PZA-R cases can be related to inactivation of the PZase, the genetic variants, including

71 single nucleotide polymorphisms (SNPs) and small deletions, are highly diverse and
72 scattered over the full length of the 561 bp *pncA* gene(4, 5). This complicates the
73 development of molecular tests, as no “hot spot region” comprising the majority of
74 mutations is observed in the *pncA* gene. Therefore, an in-depth knowledge of the
75 variants found in PZA-R strains combined with evidence-based correlation with
76 resistance phenotypes are needed to develop large-scale databases ensuring valid data
77 interpretation especially in specific local settings.

78

79 China has one of the highest rates of notified cases of *Mycobacterium tuberculosis*. In
80 2012, the Chinese Health Authorities reported a tuberculosis (TB) incidence of 39.5
81 cases per 100,000 people nationwide and that 2.3% of primary TB cases were
82 multidrug resistant (MDR-TB) cases(6). According to the National Laboratory
83 Surveillance System for Resistance in TB, 4,170 isolates from notified cases were
84 identified to be resistant to both isoniazid and rifampin. Despite the high burden of
85 MDR-TB, the magnitude of PZA resistance is largely unknown in China. Since PZA is
86 likely to remain a central component in the treatment of tuberculosis, it is critically
87 important to understand the epidemiology of PZA resistance as well as its associated
88 socio-demographic and clinical factors. The present study aims to fill this gap by
89 reporting the rates of PZA resistance in newly treated patients and previously treated
90 patients as well as the factors associated with PZA resistance in a population-based
91 multi-center study of tuberculosis in China.

92

93 MATERIALS AND METHODS

94 *Settings*

95 Four counties from Zhejiang, Jiangsu and Sichuan provinces were selected as study
96 sites. The population of these regions ranged from 490,000 to 3,260,000. The incidence
97 rate of TB cases ranged from 38.2/100,000 to 78.8/100,000 and the case mortality rate
98 ranged from 0.29% to 0.31% in 2011. The Directly Observed Treatment, Short-course
99 (DOTS) strategy has been implemented since the early 1990s in all the study sites. All
100 study sites were designated TB health facilities for TB diagnosis, treatment and
101 management. As recommended by the DOTS strategy, a standardized treatment
102 regimen is routinely applied for drug sensitive TB cases.

103 *Study design*

104 A population-based, STROBE-ME (STrengthening Reporting of OBservational studies
105 in Epidemiology-Molecular Epidemiology)-compliant cross-sectional study was
106 conducted from January 2011 to December 2013. Patients were enrolled if they were: 1)
107 diagnosed as pulmonary tuberculosis patients at the local TB designated health
108 facilities during the study period; 2) aged above 18 years old; 3) had TB culture
109 positive sputum; 4) gave written consent for their clinical data and specimens to be
110 used for further research. The sputum was collected in a sterile screw-top plastic
111 container and sent to the local TB dispensaries for microbiological culture and then
112 sent to the up-level prefectural TB laboratory for drug susceptibility testing of the

113 first-line drugs including PZA. The isolates were cultivated on Löwenstein-Jensen
114 medium (L-J medium) and forwarded to the School of Public Health, Fudan University
115 for the molecular analysis. Data regarding demographic characteristics (age, gender
116 and geographic origin of the patient), smear microscopy were obtained by
117 questionnaire interviews and reviews of the medical records. The patients'
118 identification information was replaced by a unique code in data input and analysis.
119 The questionnaires, informed consent forms and study protocols were approved by the
120 ethics committee of the School of Public Health, Fudan University.

121 ***Identification of strains and drug susceptibility testing***

122 Identification of the Mtb isolates was performed using standard microbiological tests
123 (niacin accumulation test and nitrate reduction test). Susceptibility to first-line
124 anti-tuberculosis drugs including isoniazid(INH), rifampicin(RIF), ethambutol(EMB)
125 and streptomycin (STR), were tested using the proportion method on L-J medium (INH,
126 0.2 mg/L; RIF, 40.0 mg/L; EMB, 2.0mg/L;STR, 4.0mg/L) according to the standard
127 procedure recommended by World Health Organization (WHO) guidelines (7, 8). The
128 strains were examined for their susceptibility to 100 mg/L of PZA using a MGIT
129 PZA(Nippon Becton-Dickinson Co., Ltd., Tokyo, Japan) antimicrobial susceptibility
130 testing assay, as previously described (9). For quality assurance, the DST was repeated
131 for 10% of the isolates by an external technician from Shanghai municipal CDC who
132 had passed the WHO's external quality control assurance with a consistency of over
133 95%. No discrepancies were recorded. MDR was defined as resistance to at least

134 isoniazid and rifampicin. Other drug resistance (ODR) was defined as resistance to at
135 least one of the four first-line anti-TB drugs (INH, RIF, EMB, STR) except MDR.
136 New cases were defined as cases with no more than one-month history of anti-TB
137 treatment. Previously treated cases were defined as cases with more than one-month
138 history of anti-TB treatment. (10)

139 ***MIC testing of PZA resistant isolates***

140 Furthermore, MIC tests for PZA resistant isolates were performed using the
141 Mycobacterial Growth Indicator Tube (MGIT) 960 platform according to the
142 manufacturer's standard protocol (11). Briefly, bacterial suspensions were transferred
143 to MGIT tubes containing serial PZA dilutions ranging from 64–1024 mg/L for PZA.
144 Control tubes without any antibiotics were inoculated with undiluted and 1:10 diluted
145 bacterial suspensions. The MIC was defined as the first antibiotic concentration that
146 showed less growth than the 1:10 diluted control of the corresponding strain, i.e. the
147 lowest concentration of the drug that inhibited more than 90% of the bacterial
148 population. Duplicates of the Mtb H37Rv reference strain were included in each run as
149 an inter- and intra-replication quality control. A MIC value of more than 500 mg/L was
150 defined as high level resistance to PZA (4, 12).

151 ***Molecular Characterization of PZA resistance***

152 According to Jureen et al.(13), the 561bp *pncA* gene, along with additional regions of
153 approximately 200 bp up- and downstream of the gene, was sequenced using the

154 *pncA*_F3 (AAGGCCGCGATGACACCTCT) and *pncA*_R4 (GTGTCGTAGAAGCG
155 GCCGAT) primers. These primers were used in a standard PCR to give a template for
156 the subsequent sequencing reactions. The *pncA*_F3 and *pncA*_R4 primers, as well as
157 the P3-F (ATCAGCGACTACCTGGCCGA) and P4-R
158 (GATTGCCGACGTGTCCAGAC) primers, were used to subdivide the PCR fragment
159 into two overlapping bidirectional sequencing reaction fragments. The sequencing
160 reactions were performed using a BigDye Terminator cycle sequencing kit and were
161 later run on a 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, CA).
162 Retrieved sequences were analyzed with the (ClustalW) vector NTI Advance (version
163 9) software (InfoMax, Inc.) using the wild-type H37Rv strain's *pncA* gene (Rv2043c)
164 as the master reference.

165 ***Family strain determination***

166 Spoligotyping was performed by using commercial kits from Isogen Bioscience BV
167 (Maarssen, The Netherlands). Comparison of spoligotyping patterns with the SpolDB4
168 database, identified Mtb isolates belonging to the Beijing family, i.e. defined as strains
169 that only hybridized to the last nine spacer oligonucleotides (spacers 35 to 43) (14).

170 ***Statistical analysis***

171 Sensitivity was defined by the proportion of PZA resistant isolates harboring a
172 non-synonymous *pncA* mutation, while the specificity was determined by the
173 proportion of PZA susceptible isolates harboring a wild type *pncA* gene. An

174 uncorrected Chi square test or Fisher exact test was used to compare the distributions
175 of low-level and high-level MIC among mutations in different *pncA* regions. The area
176 under the receiver operating characteristic curve (ROC-AUC) was calculated to assess
177 the performance of *pncA* mutations in predicting PZA resistance, yielding values from
178 0.5 (no predictive power) to 1.0 (perfect prediction). Sensitivity, specificity and
179 ROC-AUC were calculated by OPENEPI
180 (<http://www.openepi.com/DiagnosticTest/DiagnosticTest.htm>). Statistical analysis of
181 risk factors were performed using SPSS software (version19.0). $P \leq 0.05$ was
182 considered statistically significant. Association between studied variables and PZA
183 resistance were calculated using odds ratios (ORs) with 95% confidence intervals (CIs)
184 respectively by univariate and multivariate analysis in a Binary Logistic Regression
185 model.

186 **RESULTS**

187 ***Demographic and clinical characteristics of patients***

188 From 2011 to 2013, a total of 912 pulmonary TB patients were diagnosed and
189 registered at the local TB dispensaries in the 4 study sites. Of the 912 sputum samples
190 collected from these patients, 878 (96.3%) were included in the present study, while
191 34(3.7%) were excluded due to culture-negativity or lack of drug resistance profiles for
192 the first-line anti-TB drugs. Among the included 878 patients, 608(69.2%) were male,
193 with an average age of 47(47.3±19.2) years. Additionally, 185(21.1%) had a prior
194 treatment history and 227(25.9%) had cavities present in their lungs. There were no

195 statistically significant differences between the subjects from the four counties in terms
196 of demographic (gender and age) or clinical characteristics (prior treatment history,
197 sputum smear positive and cavity). (Table 1)

198 ***Drug susceptibility profile***

199 In total, 400 (45.6%) isolates were resistant to at least one of the four first-line anti-TB
200 drugs with 129 (14.7%) being simultaneously resistant to INH and RIF (MDR). Using
201 the MGIT 960 system, PZA resistance was observed in 147(16.7%) of the 878 isolates,
202 and ranged from 16.0% to 17.3% in the four studied sites. By comparison, the MDR
203 isolates showed a significantly higher proportion of PZA resistance rates compared to
204 the drug sensitive isolates (30.2%.vs.7.7%, $p<0.001$), while higher PZA resistance
205 were also observed in other drug resistant isolates compared to the drug sensitive
206 isolates (26.2% vs.7.7%, $p<0.001$).

207 ***Molecular characterization of pncA gene***

208 Sequencing of the *pncA* gene revealed that 136(136/878, 15.5%) isolates harbored
209 sequence alternations including 133 non-synonymous substitutions and 3 large
210 fragment insertions or deletions. Additionally, one isolate carried a synonymous
211 mutation (Ala36Ala). All substitutions occurred between nucleotide-11 and codon 180.
212 Distribution of the substitutions are shown in Figure 1. Nucleotide-11, located in the
213 putative promoter region, harbored the most frequent substitutions (9/133, 6.8%) while
214 codon 57 and codon 94 had the second most frequent mutation (7/133, 5.3%). Overall,

215 the distributions of mutations were quite comparable between isolates with PZA
216 mono-resistance and PZA resistance with MDR. (Figure 1)

217

218 In order to investigate the role of specific *pncA* mutations in PZA resistance, MICs
219 were determined for each *pncA* substitution (Figure 2). For the 133 isolates with
220 non-synonymous substitutions in *pncA*, 72.9% (97/133) had a high-level MIC
221 (>500mg/L) (Supplementary Table S1). Different MICs were seen for the same amino
222 acid change in the 7 typical substitutions including promoter (A-11G), Trp68Cys,
223 Thr76Pro, Phe94Leu, Val130Ala, Thr135Pro and Val163Gly. However among them,
224 only genetic mutation in codon 94 were simultaneously associated with both low and
225 high level of PZA resistance. In addition, 71 of 97 highly PZA resistant
226 (MIC >500mg/L) isolates contained a substitution in one of the following regions:
227 codon 51-76, codon 130-142 and codon 163-180, while 58 of 97 demonstrated an
228 extremely high-level MIC (MIC $\geq$ 1024mg/L). The *pncA* mutations also concentrated
229 in two other regions (codon 94 to 97 and nucleotide -11 to codon 16) where the
230 corresponding MICs demonstrated a relatively low level of PZA resistance (Figure 2).
231 A comparison of MIC distribution (low-level and high-level) in the five regions
232 mentioned above demonstrated statistical difference ($p<0.001$) (Table 2).

233

234 The performance of *pncA* sequencing data to predict PZA resistance was evaluated and
235 compared to the result obtained from the phenotypic MGIT 960 system (Table 3).

236 Overall, 131 non-synonymous substitutions were found in PZA resistant isolates and 5

237 were found in PZA susceptible isolates. Molecular diagnosis currently identified 89.1%
238 of PZA resistance. For MDR isolates and Beijing family isolates, the sensitivity
239 reached 94.9% and 89.7%, respectively. Performance was comparable between isolates
240 of the Beijing family (sensitivity: 89.7%, 95%CI: 83.46%-93.77%) and non-Beijing
241 family (Sensitivity: 81.8%, 95%CI: 52.30%-94.86%), and between MDR-TB
242 (Sensitivity: 94.9%, 95%CI: 83.11%-98.58%) and drug susceptible TB (Sensitivity:
243 91.9%, 95%CI: 78.70%-97.20%). Furthermore, the sensitivity of *pncA* gene mutations
244 to predict PZA resistance ranged from 81.8% to 94.7% in the studied sites, showing no
245 statistically difference based on the 95%CI of the sensitivity and specificity.

246 ***Socio-demographic and clinical characteristics associated with PZA resistance***

247 In primary univariate analysis (Table 4), age, prior treatment history and Beijing
248 family genotype were found to be associated with PZA resistance; previous treatment
249 history predicted a higher hazard of PZA mono-resistance. Age, prior treatment history,
250 cavity and Beijing family genotype were positively associated with PZA resistance in
251 MDR-TB patients.

252
253 In a multivariable analysis, age, prior treatment history and Beijing family were
254 retained in the model associated with PZA resistance, while in addition to the above
255 variables, presence of cavities was also retained in the model of PZA-R/MDR-TB and
256 age was removed from the model of PZA mono-R. Prior treatment history was a
257 significant predictor of high risk for PZA resistance ($p<0.001$; OR: 3.30; 95%CI:
258 2.131-5.101) both for PZA mono-resistance ($p<0.005$, OR: 2.86; 95%CI: 1.363-6.015)

259 and PZA-r/MDR-TB($p<0.001$, OR: 6.47; 95%CI: 3.186-13.15). Age was found to be
260 associated with PZA-R/MDR-TB ($p=0.024$; OR: 1.02; 95%CI: 1.003-1.042) and PZA
261 resistance ($p=0.012$; OR: 1.01; 95%CI: 1.003-1.023), while Beijing family strain were
262 associated with PZA resistance ($p=0.019$; OR: 2.25; 95%CI: 1.140-4.451).
263 Additionally, MDR-TB patients with cavitation were more likely to be infected with
264 PZA resistant strains ($p=0.008$; OR: 2.64; 95%CI: 1.296-5.391).

265

266 **DISCUSSION**

267 It has been widely accepted that PZA resistance could compromise anti-TB treatment,
268 particularly MDR-TB treatment (15-17); however, there is little epidemiological data
269 on PZA resistance, especially in high MDR-TB burden areas. In China, in contrast to
270 earlier hospital-based studies or those conducted in a single region (18), the present
271 study was multi-center, to better understand the epidemiological situation of PZA
272 resistance across China.

273

274 This is the first reported population-based study of PZA resistance among PTB cases in
275 China. PZA susceptibility testing by BACTEC MGIT 960 revealed that 16.7%
276 (147/878) of the studied patients were PZA resistant. Simultaneously, a higher level of
277 PZA resistance (30.2% [39/129]) was found in the MDR-TB isolates, which is
278 consistent with previously reported studies with PZA resistant rates ranging from 36%
279 to 54% among MDR-TB patients (18-20). A significantly higher proportion of PZA
280 resistance was observed in MDR cases compared to drug susceptible cases

281 (30.2%.vs.7.7%, $p<0.001$). Unfortunately, the overall PZA mono-resistance rate was
282 7.7%, a rate higher than that reported in most areas with low TB incidence. This might
283 have two explanations. Firstly, the PZA mono-resistance rate could be explained by the
284 high proportion of previously treated patients included in the present study. Secondly,
285 this could be due to an endemic spread of PZA mono-resistant strain, which has been
286 observed elsewhere (21) or a combination of both. This suggests that PZA resistance
287 has been established throughout China and that PZA susceptibility testing should be
288 prioritized, especially among MDR-TB patients (even in areas with low prevalence of
289 MDR-TB).

290
291 For the 147 strains phenotypically resistant to PZA, mutations in the *pncA* gene were
292 detected in 131 cases, indicating a sensitivity of 89.1%. In addition, as previous studies
293 demonstrated, mutations were scattered throughout the *pncA* gene. Among the 731
294 phenotypically PZA-susceptible isolates, we found 726 isolates with a wild type *pncA*,
295 resulting in a specificity of 99.3%. Most importantly, the diagnostic performances of
296 *pncA* mutations were similar irrespective of genotype (Beijing family vs. non-Beijing
297 family), MDR and geographic area of origin. The most frequent alteration was
298 observed in the putative promoter (nucleotide position -11), which might influence the
299 initiation of protein synthesis and subsequent binding of the ribosome to the *pncA*
300 mRNA (22). Additionally, the frequent mutation found at codon 57(7/130, 5.4%) were
301 reported to be involved in the suppression of metal iron (Mg^{2+}/Fe^{2+}) binding, leading
302 to a drastic decrease of PZase activity (23), while the mutation at codon

303 94(Phe94Leu/Cys/Ser) has been shown to be engaged in the hydrophobic core which
304 would decrease the stability of the PZase. To our knowledge, 4 SNPs (7 isolates) are
305 reported here for the first time (Gly16Ser, Asp33Ala, Thr153Ile, Val163Gly),
306 indicating the highly diverse patterns of *pncA* mutations. The isolates carrying these
307 novel mutations ($n=7$) also had a high-level MIC and the role of these mutations needs
308 to be studied further.

309

310 Additionally, due to the clinical significance of MIC in TB treatment (24), we also
311 related MIC levels to the *pncA* mutations, in an attempt to search for certain regions or
312 substitutions that led to different levels of PZA resistance. In total, 97 of 133 PZA
313 resistant isolates demonstrated a relatively high MIC (>500 mg/L), suggesting high
314 level PZA resistance. Most interestingly, the majority of those cases were associated
315 with mutations in three specific regions of the *pncA* gene (codon 51-76, codon 130-142
316 and codon163-180). As previously reported (25), these three *pncA* regions related to
317 high-level PZA resistance might be associated with the activity of PZase. As structural
318 models of the PZase have revealed, these three specific regions were located in loop 4,
319 loop 7 and loop 10, which all have been proven to be the part of PZase active site
320 region (loop 7) and the metal coordination site (loop4) respectively (26). Our results
321 were consistent with those studies and suggested that substitutions influencing the
322 function of the PZase catalytic site could result in a high level of PZA resistance.
323 Additionally, we also observed that for 7 codons, the same substitution led to different
324 MIC values and this that other mechanisms apart from *pncA* mutation could also

325 influence the level of PZA resistance. Given the genetic profile of *pncA* in the PZA
326 resistant isolates, a method based on these specific *pncA* regions might be of particular
327 interest for a rapid molecular test of PZA resistance.

328

329 Risk factors associated with PZA resistance among cases of *Mtb* are less well
330 understood, especially in TB high-burden countries. In the present study, we did find
331 that PZA mono-resistance was exclusively associated with previous treatment history.
332 Additionally, given the important role of PZA in MDR-TB treatment, we also
333 investigated the factors associated with PZA resistance among MDR-TB patients. In a
334 multivariable analysis, factors significantly associated with MDR-PZA resistance
335 included age and lung cavitation. Meanwhile despite the association between Beijing
336 family and drug resistance has remained controversial with discrepant findings in
337 China (27-29), in the present study, the association between Beijing family and PZA
338 resistance was observed in other 1st line drug resistant strains rather than in isolates
339 with PZA mono-resistance and MDR. This may rule out the possibility that the Beijing
340 family in China has a propensity towards development of resistance to
341 PZA. Alternatively, a secondary explanation could be that the risk of PZA resistance
342 might be further increased within Beijing family strains due to other host
343 characteristics relevant to other 1st line drug resistance (age and previous treatment
344 history). According to these observations and also for the development of novel
345 method for rapid identification of Beijing family strains (30), we would recommend
346 testing for PZA susceptibility in previously treated patients and especially those

347 infected with strains of the Beijing family if possible.

348

349 Our study was subject to several limitations. Since PZA susceptibility testing has not
350 being routinely conducted in China, we decided to focus on four study sites with the
351 ability to perform these tests. For the other areas that do not routinely test for PZA
352 resistance, the prevalence may have been underestimated due to selection bias.
353 Considering this limitation, we selected four areas with different geographic and
354 socio-economic characteristics in China. We therefore think that the findings can give
355 a reliable picture of the real situation. In addition, the phenotypic testing of PZA
356 resistance was performed in the BACTEC MGIT 960 system which is the reference
357 method recommended by WHO. The risk of false results using this methodology is
358 however well known (31), but by randomly choosing 10% of the sputum culture
359 samples and repeating the phenotypic DST with 100% consistency (compared to the
360 previous testing), we estimate that our MGIT 960 method is reliable.

361

362 **Conclusion**

363 The significantly higher prevalence of PZA resistance in MDR-TB isolates (30%)
364 highlights the need for PZA susceptibility testing in patients infected with MDR-TB
365 and since PZA is an essential part of anti-MDR-TB treatment PZA resistance is
366 undoubtedly an important public health issue which needs to be addressed.
367 Additionally, the reliable performance of *pncA* mutations in detecting PZA resistance

368 as well as the association between specific regions of *pncA* and high-level PZA
369 resistance, show the value of a rapid molecular test for PZA resistance.

370

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Table1. Demographic and clinical characteristics of patients and drug-resistant patterns at diagnosis in the four studied sites

	GY	DQ	GJ	RO	Total		
Variables	(n=254)	(n=216)	(n=237)	(n=171)	(n=878)	χ^2	<i>p</i>
Male(Gender)	184(72.4)	152(70.4)	157(66.2)	115(67.3)	608(69.2)	2.668	0.446
Age(Mean±SD)	45.3±19.5	48.2±18.0	47.5±19.5	49.1±19.9	47.3±19.2	1.626 ^a	0.182
Prior treatment history	56(22.0)	41(19.0)	52(21.9)	36(21.1)	185(21.1)	0.820	0.845
Sputum Smear positive	159(62.6)	140(64.8)	143(60.3)	91(53.2)	533(60.7)	5.945	0.114
Cavity	70(27.6)	47(21.8)	62(26.2)	48(28.1)	227(25.9)	2.724	0.436
1st-line Drug susceptibilities							
DS	116(45.7)	134(62.0)	128(54.0)	100(58.5)	478(54.4)	22.468	0.001 ^b
MDR	34(13.4)	25(11.6)	40(16.9)	30(17.5)	129(14.7)		

ODR	104(40.9)	57(26.4)	69(29.1)	41(24.0)	271(30.9)		
PZA susceptibilities							
PZA resistance	44(17.3)	37(17.1)	38(16.0)	28(16.4)	147(16.7)	0.187	0.980
PZA mono-R	12(4.7)	13(6.0)	6(2.5)	6(3.5)	37(4.2)	3.779	0.286
PZA-R/MDR	8(3.1)	9(4.2)	11(4.6)	11(6.4)	39(4.4)	2.657	0.448

487 Note: DS: Drug susceptible; MDR: Multidrug-resistant; ODR: Other drug resistant: PZA: pyrazinamide

488 ^a F value for ANOVA test

489 ^b $p < 0.05$

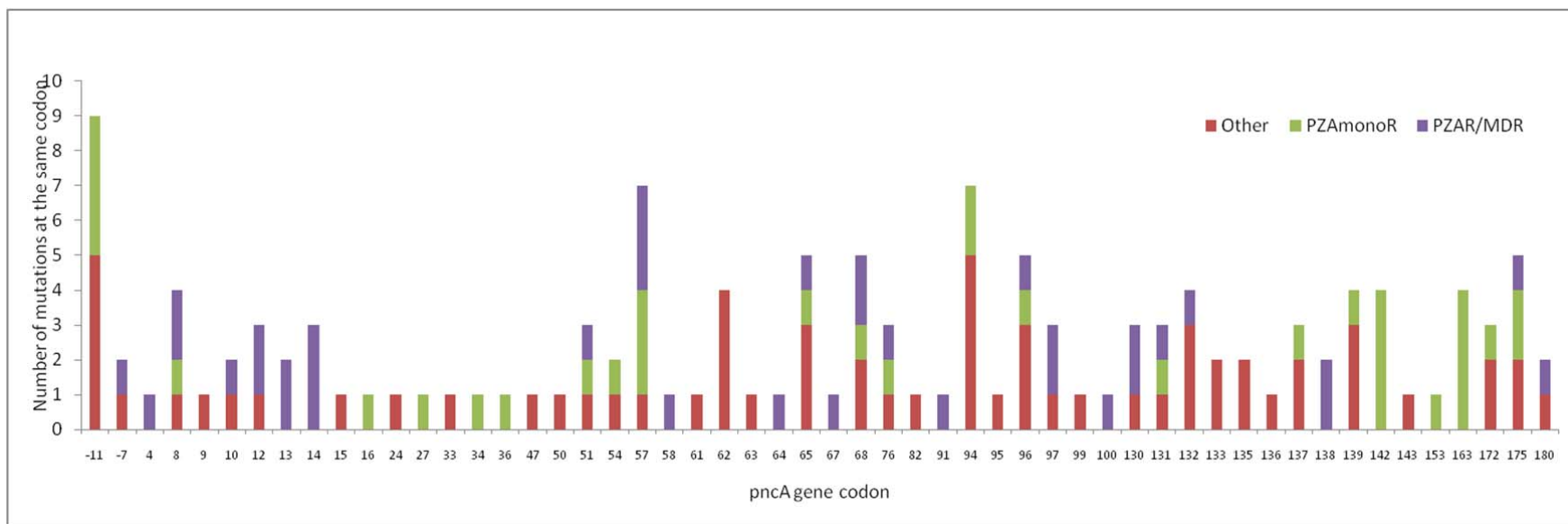
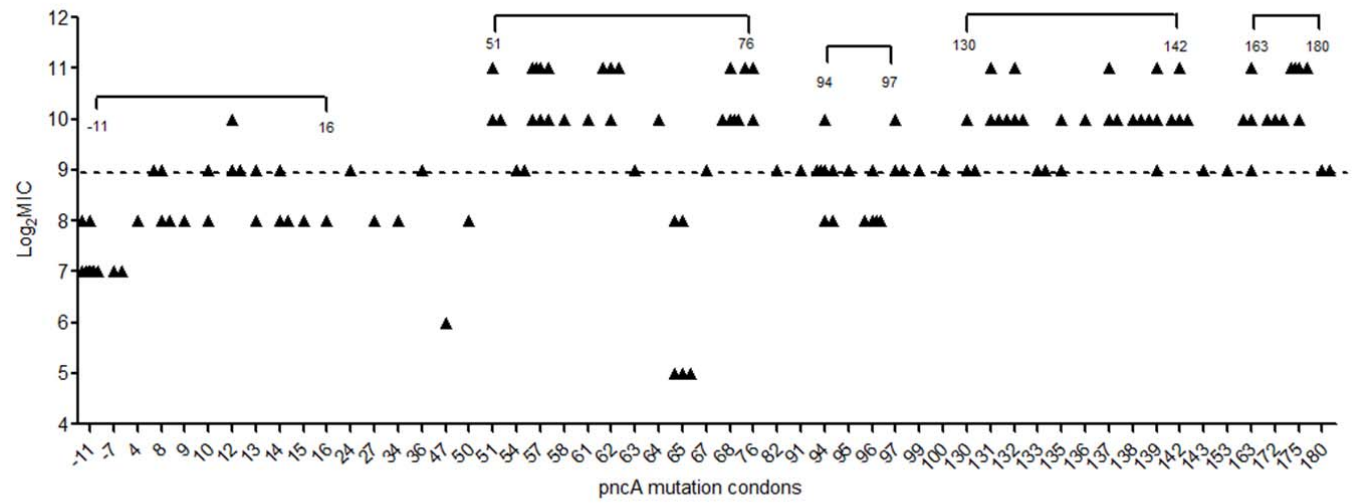


Figure 1. Number of mutations at each codon in the *pncA* gene among different resistance patterns

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494 Figure 2. The frequency and MICs of PZA among the isolates with mutations in *pncA* gene. The MIC is presented for each isolate and the
495 symbols above the dotted line indicate high level PZA resistance (MIC > 500 mg/L).

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497

Table2. Regions of *pncA* correlated with low or high-level PZA resistance

	Total	low-level PZA resistance(MIC≤500mg/L)	high-level PZA resistance(MIC>500mg/L)	<i>p</i> ^a
Codon 51-76	34	5(14.7)	29(85.3)	
Codon 130-142	28	0(0.0)	28(100.0)	
Codon 163-180	14	0(0.0)	14(100.0)	<0.001
Codon 94-97	16	6(37.5)	10(62.5)	
Nucleotide -11 to codon 16	29	21(72.4)	8(27.6)	

498 Note: PZA: pyrazinamide

499 ^a Calculated by Fisher exact test

Table 3.Comparison of non-synonymous *pncA* mutations with PZA resistance results based on BATECT MGIT 960

PZA susceptibility	No. of isolates with:		Sensitivity % (95%CI)	Specificity %(95%CI)	ROC-AUC(95%CI)
	<i>pncA</i> nonsynonymous	<i>pncA</i> wide type or synonymous			
	mutations	mutations			
Overall, n=878					
Resistant	131	16	89.1(83.05, 93.19)	99.3(98.41,99.71)	0.94(0.925,0.957)
Susceptible	5	726			
MDR-TB, n=129					
Resistant	37	2	94.9(83.11,98.58)	96.7(90.65,98.86)	0.96(0.907,0.985)
Susceptible	3	87			
ODR-TB, n=271					
Resistant	60	11	84.5(74.35, 91.12)	99.0(96.43, 99.73)	0.92(0.878,0.947)
Susceptible	2	198			

DS, n=478

Resistant	34	3	91.9(78.70, 97.20)	100.0(99.14, 100.00)	0.96(0.938,0.975)
Susceptible	0	441			

Beijing strain, n=739

Resistant	122	14	89.7(83.46,93.77)	99.2(98.07%,99.65)	0.94(0.925,0.960)
Susceptible	5	598			

Non-Beijing strain, n=139

Resistant	9	2	81.8(52.30, 94.86)	100.0(97.09, 100.00)	0.91(0.849,0.951)
Susceptible	0	128			

GY, n=254

Resistant	36	8	81.8(68.04,90.49)	99.0(96.59,99.74)	0.90(0.861,0.938)
Susceptible	2	208			

DQ, n=216

Resistant	33	4	89.2(75.29,95.71)	98.3(95.19,99.43)	0.94(0.897,0.966)
Susceptible	3	176			
GJ, n=237					
Resistant	36	2	94.7(82.71,98.54)	100.0(98.11,100.00)	0.97(0.944,0.990)
Susceptible	0	199			
RO, n=171					
Resistant	26	2	92.9(77.35,98.02)	100.0(97.38,100.00)	0.96(0.924,0.987)
Susceptible	0	143			

501 Note: DS: drug susceptible; MDR: multidrug-resistant; ODR: other drug resistant; PZA: pyrazinamide; ROC-AUC: The area under the receiver
502 operating characteristic curve.

Table4. Socio-demographic and clinical characteristics associated with PZA resistance

Risk factors	PZA Mono-R ^a N=37	PZA-R/MDR ^b N=39	PZA- R N=147	DrugS ^c N=441	PZA mono-R VS DrugS		PZA-R/MDR VS DrugS		PZA-R VS DrugS	
	n(%)	n(%)	n(%)	n(%)	p ^d	OR(95%CI) ^d	p ^d	OR(95%CI) ^d	p ^d	OR(95%CI) ^d
Gender(Male)	30 (81.1)	26 (66.7)	106 (72.1)	301 (68.3)	0.110	1.99 (0.855-4.649)	0.838	0.93 (0.464-1.864)	0.381	1.20 (0.796-1.816)
Age	51.1 ±21.3	54.4 ±20.3	54.4 ±20.3	46.8 ±18.8	0.189	1.01 (0.994-1.030)	0.018 ^c	1.02 (1.004-1.039)	0.003 ^c	1.02 (1.005-1.025)
Prior	12	20	53	62	0.004 ^c	2.93	<0.001 ^c	6.44	<0.001	3.45

treatment	(32.4)	(51.3)	(36.1)	(14.1)	(1.402-6.143)	(3.250-12.74)	°	(2.241-5.302
history								)
Sputum								1.13
smear	23	25	91	260	1.14	1.24		
Positive	(62.2)	(64.1)	(61.9)	(59.0)	0.703	0.531	0.528	(0.771-1.659
					(0.573-2.282)	(0.629-2.457)		)
								1.02
Cavity	10	18	38	112	1.09	2.52		
	(27.0)	(46.2)	(25.9)	(25.4)	0.827	0.007°	0.913	(0.668-1.570
					(0.511-2.318)	(1.295-4.896)		)
								2.41
Beijing F	35	38	136	369	3.42	7.42		
	(94.6)	(97.4)	(92.5)	(83.7)	0.096	0.050°	0.009°	(1.242-4.287
					(0.803-13.52)	(1.002-44.87)		)

504 Note: DS: drug susceptible; MDR: multidrug-resistant; ODR: other drug resistant; PZA: pyrazinamide;

505 ^a PZA mono-R: isolates resistant to PZA but susceptible to isoniazid, rifampicin, ethambutol and streptomycin;

506 ^b PZA-R/MDR: isolates resistant to PZA as well as at least resistant to isoniazid and rifampicin;

507 ^c DrugS: isolates susceptible to all the 5 tested drugs (isoniazid, rifampicin, ethambutol, streptomycin and pyrazinamide);

508 ^d p and OR(95%CI) derived from the Binary Logistic Regression model;

509 ^e $p < 0.05$.

Table 5. Multivariate analysis of demographic and clinical characteristics associated with PZA resistance

	β	p	OR(95%CI) ^a
PZA mono-R vs Drug S			
Prior Treatment History	1.052	0.005 ^b	2.86(1.363-6.015)
Beijing Family	1.186	0.109	3.28(0.766-14.00)
PZA-R/MDR vs Drug S			
Age	0.022	0.024 ^b	1.02(1.003-1.042)
Prior Treatment History	1.867	<0.001 ^b	6.47(3.186-13.15)
Cavity	0.972	0.008 ^b	2.64(1.296-5.391)
Beijing Family	1.849	0.074	6.35(0.838-48.14)
PZA-R vs Drug S			
Age	0.013	0.012 ^b	1.01(1.003-1.023)
Prior Treatment History	1.193	<0.001 ^b	3.30(2.131-5.101)

Beijing Family	0.812	0.019 ^b	2.25(1.140-4.451)
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511 Note: DS: drug susceptible; MDR: multidrug-resistant; ODR: other drug resistant; PZA: pyrazinamide.

512 ^a OR and 95%CI was calculated in the Binary Logistic Regression model by backward selection

513 ^b $p<0.05$