Resistance To First-Line Antituberculosis Drugs And Prevalence Of \textit{pncA} Mutations In Clinical Isolates Of \textit{Mycobacterium tuberculosis} From Zunyi, Guizhou Province Of China

Zhimin Cao\textsuperscript{1}
Yuanbo Lan\textsuperscript{1}
Ling Chen\textsuperscript{1}
Min Xiang\textsuperscript{1}
Zhiyuan Peng\textsuperscript{1}
Jianyong Zhang\textsuperscript{1}
Hong Zhang\textsuperscript{1,2}

\textsuperscript{1}Tuberculosis Division, Department of Respiratory and Critical Care Medicine, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou 563003, People’s Republic of China; \textsuperscript{2}Department of R & D, Z-BioMed, Inc, Rockville, MD, 20855, USA

\textbf{Background:} China is one of the high-burden countries for multidrug-resistant tuberculosis (MDR-TB), and pyrazinamide is one of the anti-TB drugs used for the shorter MDR-TB treatment regimen. The aim of this study was to determine the correlation between \textit{pncA} gene mutations and resistance to four first-line anti-TB drugs as well as treatment history in clinical isolates of \textit{Mycobacterium tuberculosis}.

\textbf{Patients and methods:} \textit{M. tuberculosis} clinical isolates were collected from 318 in-patients with smear-positive TB between October 2008 and September 2016 at a major hospital in Zunyi, Guizhou Province of China, and used for drug susceptibility testing against four first-line anti-TB drugs. Genomic DNA extracted from clinical isolates was used for PCR amplification and DNA sequencing of the \textit{pncA} gene.

\textbf{Results:} Among 318 clinical isolates, 129 (40.6%), 170 (53.5%), 66 (20.8%) and 109 (34.3%) were resistant to rifampicin, isoniazid, ethambutol and streptomycin respectively. In addition, 124 clinical isolates were MDR-TB and 71.8% of them were previously treated cases. Sequencing results showed that 46.8% of MDR-TB and 2.2% of drug susceptible isolates harbored a \textit{pncA} mutation, and 52 types of \textit{pncA} mutations were detected from 64 isolates. The prevalence of \textit{pncA} mutations in isolates resistant to first-line anti-TB drugs and previously treated TB cases was significantly higher than that in drug-susceptible isolates and new cases of TB.

\textbf{Conclusion:} High prevalence of \textit{pncA} mutations in clinical isolates of \textit{M. tuberculosis} from Zunyi, Guizhou Province of China, is correlated with resistance to four first-line anti-TB drugs, MDR-TB and previously treated TB cases.

\textbf{Keywords:} \textit{Mycobacterium tuberculosis}, pyrazinamide, drug resistance, \textit{pncA} mutation

\textbf{Introduction}

Tuberculosis (TB), a chronic infectious disease, caused about 1.6 million deaths globally in 2017, and China is one of the high-burden countries for multidrug-resistant tuberculosis (MDR-TB).\textsuperscript{1} The recommended treatment for drug-susceptible TB is a six-month first-line regimen including two months of isoniazid, rifampicin, pyrazinamide and ethambutol followed by four months of isoniazid and rifampicin.\textsuperscript{1} Pyrazinamide (PZA or Z) is also one of the antituberculosis drugs used for the shorter MDR-TB treatment regimen with sterilizing and bactericidal activity. Pyrazinamide, a synthetic nicotinamide analog, has to be converted from a pro-drug to its active form...
pyrazinamide (PZA) in the cytoplasm by the nicotinamide/ pyrazinamidase (PZase). Both PZA and POA have been shown to have different enzyme targets interfering with diverse biochemical pathways involved in the mycobacterial energy metabolism, lipid synthesis and membrane transport. Previous studies have shown that PZA resistance in *Mycobacterium tuberculosis* is mainly caused by various mutations in the PZase coding gene, *pncA*, or its promoter region leading to a decrease/loss of PZase activity or reduced expression of *pncA* gene. Drug susceptibility testing (DST) against PZA is not routinely performed in most resource-limited regions due to its complexity, inconsistency and high cost. Therefore, an alternative *pncA* gene-sequencing method was developed to detect mutations in the *pncA* gene and to rapidly screen for PZA susceptibility with 90.9% of sensitivity and 100% of specificity. Currently, DNA sequencing of the *pncA* gene is the proposed reference method for DST against PZA because there is no WHO recommended rapid method. In a systematic review of mutations reported in 61 studies, 641 unique mutations in 171 out of 187 codons of the *pncA* gene (561 bp) and its promoter region from 2,760 PZA-resistant isolates and 96 mutations from 3,329 PZA-susceptible isolates were recognized. In another study, more than 300 mutations were identified through in vitro saturating mutagenesis of the *pncA* gene which mapped throughout the entire *pncA* coding region and conferred resistance to PZA. It was reported from a multicountry surveillance project involving the detection of *pncA* mutations that PZA resistance was significantly associated with rifampicin-resistant TB (RR-TB) cases. Other studies showed that 74% of PZA-resistant TB isolates from Pakistan, more than half of MDR/RR-TB isolates from Sub-Sahara Africa countries and Georgia, and 37.5% of PZA-resistant TB isolates from Yunnan, China, had a mutation in the *pncA* gene. However, it was unclear whether similar correlations could be observed in clinical isolates of *M. tuberculosis* from Zunyi, Guizhou Province of China. A total of 318 in-patients with smear-positive TB were registered at the Tuberculosis Division of Respiratory and Critical Care Medicine of the hospital. Clinical isolates of *M. tuberculosis* were collected from the sputum and bronchoscope brush specimens of 318 TB patients as a part of routing hospital laboratory procedures in a period from October 2008 to September 2016. These clinical isolates were not specifically collected for this study; however, patient identifiers were removed from clinical isolates prior to the initiation of this study. Collected clinical specimens were cultured on Löwenstein–Jensen solid slants by following the procedures recommended by WHO and grown colonies were identified to the species level using 2-thiophene carboxylic acid and para-nitrobenzoic acid selective media.

Drug Susceptibility Testing

Drug susceptibility testing (DST) of *M. tuberculosis* clinical isolates was conducted at the Laboratory of Respiratory Medicine in the hospital, which was certified by the Chinese Center for Disease Control and Prevention (CCDC). The proportion method on Löwenstein–Jensen solid slants was used for DST against four first-line anti-TB drugs purchased from Sigma-Aldrich (St. Louis, MO, USA). The critical drug concentrations were: 40 μg/mL of rifampicin (RIF), 0.2 μg/mL of isoniazid (INH), 4 μg/mL of streptomycin (SM), and 2 μg/mL of ethambutol (EMB). The standard *M. tuberculosis* strain H37Rv was obtained from the China CDC and used as a control for all the tests. The critical proportion of four first-line anti-TB drugs was one percent and multidrug resistance was defined as resistance to both RIF and INH.

DNA Extraction And PCR Amplification

Genomic DNAs were extracted from 318 clinical isolates of *M. tuberculosis* and the standard H37Rv strain using the cetyltrimethylammonium bromide (CTAB) method. Two oligonucleotide primers, pncA-F (5'-GCTGTCATGTTCGCGATCG-3') and pncA-R (5'-GCTTGCGCGGAGGCTCCA-3'), were designed by using the Web Primer website (http://seq.yeastgenome.org/cgi-bin/web-primer), and used for PCR amplification of the *pncA* gene with purified genomic DNA as a template. The PCR reaction mixture (25 μL) contained 12.5 μL of 2× Taq Master Mix (Beijing TIANGEN Biotech Co., Ltd., China), 0.5 μL of DNA template, and 0.5 μL of each primer pair at a concentration of 20 mM. The thermal cycling conditions were 5 min at 94°C for denaturation followed by 30 cycles of 94°C for 1 min for

Patients And Methods

Patients And Clinical Isolates Of *M. tuberculosis*

This study was conducted at the Affiliated Hospital of Zunyi Medical University, a tertiary general hospital in Zunyi, Guizhou Province of China. The aim of this study was to determine the correlation between *pncA* gene mutations and resistance to four first-line anti-TB drugs, MDR-TB as well as treatment history in clinical isolates of *M. tuberculosis* from a major hospital in Zunyi, Guizhou Province of China.
denaturation, 60°C for 1 min for annealing, and 72°C for 1 min for amplification; and a final extension of 10 min at 70°C. The amplified PCR products (719bp, genome sequences from 2289345 to 2288626) were verified by agarose gel electrophoresis and sent to Shanghai Invitrogen for sequencing using primers pncA-F and pncA-R. The sequencing results were analyzed and mutations in the pncA gene were identified by aligning them with the wild-type pncA gene (GeneID: 887497) of the reference strain H37Rv using the BLAST (bl2seq) program at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data Analysis
The chi-square (χ²) test was used to evaluate the correlation between pncA gene mutations in clinical isolates of M. tuberculosis and resistance to each of the first-line anti-TB drugs as well as treatment history of TB patients. Differences with a P value less than 0.05 were determined to be statistically significant.

Results
Demographic Information About TB Patients
M. tuberculosis clinical isolates were collected consecutively from patients with active pulmonary TB at the hospital between October 2008 and September 2016. Among 318 TB patients, 196 (61.6%) were male and 122 (38.4%) were female; 187 (58.8%) were new cases and 131 (41.2%) were previously treated cases. Patients were divided into three age groups: <35 years (104, 32.7%), 35–55 years (104, 32.7%) and >55 years (110, 34.6%). The average age of patients was 49.3 years, the youngest patient was 13 years old and the oldest patient was 95 years old (Table 1).

Drug Susceptibility Testing Against First-Line Anti-TB Drugs
M. tuberculosis clinical isolates were analyzed for their drug susceptibility profiles against four first-line anti-TB drugs (RIF, INH, EMB, and SM). Among 318 collected clinical isolates, 129 (40.6%), 170 (53.5%), 66 (20.8%) and 109 (34.3%) were resistant to RIF, INH, EMB and SM respectively; 124 (39%) were identified as MDR-TB; 58 (18.2%) were mono-resistant/poly-drug resistant TB (MR/PDR-TB); and 136 clinical isolates (42.8%) were determined to be pan-susceptible to four first-line anti-TB drugs (Pan-S-TB) (Table 1). In addition, 35 out of 124 MDR-TB cases (28.2%), 43 out of 58 MR/PDR-TB cases (74.1%) and 109 out of 136 Pan-S-TB cases (80.1%) were new cases. Whereas, 89 out of 124 MDR-TB cases (71.8%), 15 out of 58 MR/PDR-TB cases (25.9%) and 27 out of 136 Pan-S-TB cases (19.9%) were previously treated cases (Table 1).

Detection Of pncA Mutations In M. tuberculosis Clinical Isolates
A total of 136 Pan-S-TB, 124 MDR-TB and 58 MR/PDR-TB isolates were used for DNA extraction and sequencing of the pncA gene (Table 1). Two primers, pncA-F and pncA-R, were designed to amplify a genomic DNA fragment covering the entire pncA gene plus extra 104 bp at the 5′ end and extra 54 bp at the 3′ end. Mutations in the pncA gene should not cause possible PCR negatives because both primers were located outside of the pncA coding region. The same primer set was also used for sequencing of the amplified PCR products (719bp). Analysis of sequencing results showed that 64 out of 318 clinical isolates (20.1%), including 58 MDR, three PDR and three Pan-S TB isolates had a mutation in the pncA gene (Table 2). Among 64 clinical isolates, 52 different types of pncA mutations were detected which included point mutations, insertions and deletions. The codon 131 was mutated most frequently (6 out of 64 isolates, five insertions and one point mutation Lys131Phe) followed by codon 76 with 4 isolates and codon 67 with three isolates (Table 2 and Figure 1).

Prevalence Of pncA Mutations And Resistance To First-Line Anti-TB Drugs And Treatment History
Among 124 MDR-TB isolates, 58 (46.8%) harbored a mutation in the pncA gene, whereas, only three out of 136 Pan-S-TB isolates (2.2%) had a mutation in the pncA gene and the difference was statistically significant (p<0.01) (Table 3). The prevalence of pncA mutations in isolates resistant to four first-line anti-TB drugs was higher than that in drug-susceptible isolates and the differences were also statistically significant (p<0.01): 46.5% verse 2.1% for RIF, 34.1% verse 4.1% for INH, 42.4% verse 14% for EMB, and 40.4% verse 9.1% for SM (Table 3). When treatment history of TB patients was considered, the prevalence of pncA mutations in previously treated TB cases (36.2%) was much higher than that in the new cases of TB (9%) and the difference was statistically significant (p<0.01) (Table 3).
Table 1 Demographic Characteristics And Treatment History Of 318 TB Patients

| Characteristics And Treatment History | MDR-TB (n=124) | MR/PDR-TB (n=58) | Pan-S-TB (n=136) | Total (n=318) |
|---------------------------------------|---------------|-----------------|-----------------|--------------|
|                                       | No. (%)       | No. (%)         | No. (%)         | No. (%)      |
| Gender                                |               |                 |                 |              |
| Male                                  | 75 (60.5%)    | 39 (67.2%)      | 82 (60.3%)      | 196 (61.6%)  |
| Female                                | 49 (39.5%)    | 19 (32.8%)      | 54 (39.7%)      | 122 (38.4%)  |
| Age (years)                           |               |                 |                 |              |
| <35                                   | 37 (29.8%)    | 22 (37.9%)      | 45 (33.1%)      | 104 (32.7%)  |
| 35–55                                 | 58 (46.8%)    | 14 (24.2%)      | 32 (23.5%)      | 104 (32.7%)  |
| >55                                   | 29 (42.8%)    | 22 (37.9%)      | 59 (43.4%)      | 110 (34.6%)  |
| Average                               | 42.8          | 45.8            | 56.7            | 49.3         |
| Youngest                              | 16            | 13              | 16              | 13           |
| Oldest                                | 83            |                 | 95              | 95           |
| Treatment history                     |               |                 |                 |              |
| New case                              | 35 (28.2%)    | 43 (74.1%)      | 109 (80.1%)     | 187 (58.8%)  |
| Previously treated                    | 89 (71.8%)    | 15 (25.9%)      | 27 (19.9%)      | 131 (41.2%)  |

Abbreviations: MDR-TB, multidrug-resistant tuberculosis; MR/PDR-TB, monodrug-resistant/poly-drug resistant tuberculosis; Pan-S-TB, pan-susceptible tuberculosis.

Table 2 pncA Gene Mutations Detected In 64 Clinical Isolates Of M. tuberculosis

| Nucleotide Position | Codon Change | Amino Acid Change | Nucleotide Position | Codon Change | Amino Acid Change |
|---------------------|--------------|-------------------|---------------------|--------------|-------------------|
| 11*                 | TTG4TCG      | Leu4Ser           | 289                 | GGT97AGT     | Gly97Ser          |
| 29                  | CAG10CCG     | Gln10Pro          | 289                 | GGT97CGT     | Gly97Arg          |
| 35                  | GAC12GCC     | Asp12Ala          | 304                 | GCC102ACG    | Ala102Thr         |
| 50                  | Insertion of G | FSC 17 (ins) | 309                 | TAC103TAG    | Tyr103Stop        |
| 62                  | GTA21GCA     | Val21Ala          | 314                 | Insertion of G | FSC 105 (ins) |
| 63–73               | Deletion of AA | FSC 21–25 (del) | 338                 | Insertion of G | FSC 113 (ins) |
| 85                  | CGC29TGC     | Arg29Cys          | 357                 | TGG119TGT    | Trp119Cys         |
| 124                 | CAT42AAT     | His42Asn          | 362                 | CGG121CCG    | Arg121Pro         |
| 136                 | Deletion of G | FSC 46 (del) | 391                 | Insertion of G | FSC 131 (ins) |
| 137                 | GCA46GTA     | Ala46Val          | 391                 | Insertion of GG | FSC 131 (ins) |
| 139*                | ACC47GCC     | Thr47Ala          | 391                 | GTG131TTC    | Val131Phe         |
| 160                 | CCG54GCC     | Pro54Ala          | 398                 | ATT133ACT    | Ile133Thr         |
| 161                 | CCG54CAG     | Pro54Gln          | 403                 | ACC135GCC    | Thr135Ala         |
| 172                 | TTC58GTC     | Phe58Val          | 407                 | GAT136GTT    | Asp136Gly         |
| 184                 | CCG62TCG     | Pro62Ser          | 416                 | GTG139GCC    | Val139Ala         |
| 185                 | CCG62CAG     | Pro62Arg          | 416                 | GTG139GAG    | Val139Gly         |
| 193                 | TCC65CCC     | Ser65Pro          | 422                 | CAG141CCG    | Gln141Arg         |
| 200*                | TCG67TAG     | Ser67Stop         | 423                 | CAG141CAC    | Gln141His         |
| 200                 | TCG67TTC     | Ser67Leu          | 425                 | ACG142ATG    | Thr142Met         |
| 212                 | CAT71CGT     | His71Arg          | 464                 | GTG155GAG    | Val155Glu         |
| 226*                | ACT76CCT     | Thr76Pro          | 478                 | ACA160GCA    | Thr160Ala         |
| 231                 | Insertion of G | FSC 77 (ins) | 484                 | GGT162CGT    | Gly162Arg         |
| 233                 | GCC78GAC     | Gly78Asp          | 512                 | GCC171GAG    | Ala171Glu         |
| 285                 | Deletion of TAC | FSC 95 (del) | 524                 | ATG175AGG    | Met175Arg         |
| 285                 | TAC95TAA     | Tyr95Stop         | 539                 | GTC180GCC    | Val180Ala         |
| 286                 | AAG96CAG     | Lys96Gln          | 554                 | AGC185ATC    | Ser185Ile          |

Notes: *Two isolates with the same mutation; **Three isolates with the same mutation; ***Four isolates with the same mutation.

Abbreviations: del, deletion; FSC, frame-shift codon; ins, insertion.
Discussion

Pyrazinamide is one of the major anti-TB drugs used for both the first- and second-line regimens, which plays an important role in reducing the treatment duration for drug-susceptible and drug-resistant TB.\(^1\) Drug susceptibility testing against PZA is not routinely performed, and mutations in the \(pncA\) gene or the \(pncA\) promoter region have been proved to be the main molecular mechanism causing PZA resistance in clinical isolates of \(M.\) \(tuberculosis.\)\(^3\) Therefore, detection of mutations in the \(pncA\) gene by DNA sequencing is the proposed reference method for rapid screening of PZA susceptibility.\(^5\),\(^6\) Results from a large multicenter study assessing \(pncA\) sequence variations in 1,950 clinical isolates showed that 888 (45.5%) isolates harbored 280 mutations in the \(pncA\) gene.\(^14\)

During the current study, \(M.\) \(tuberculosis\) clinical isolates were collected from 318 TB patients and the proportion of male to female patients was 61.6% to 38.4%, which was consistent with our previous study for male (62.8%) and female (37.2%) patients.\(^15\) However, the proportion of previously treated cases in this study (41.2%) was higher than that in our previous study (31%),\(^15\) probably because only in-patients were enrolled in this study. For the same reason, rates of MDR-TB in new (18.7%, 35/187) and previously treated TB cases (69.7%, 89/131) were higher than those in our previous studies\(^15\) and combined data from 53 Member States in the WHO European Region (15.7% of new and 45.3% of previously treated cases of TB were MDR-TB).\(^16\) Most patients from this study (65.4%) were 55 years of age or younger and the average age of patients was 49.3 years (Table 1).

Our PCR and sequencing results showed that the prevalence of \(pncA\) mutations in clinical isolates resistant to each of the four first-line anti-TB drugs (RIF, INH, EMB and SM), MDR-TB and previously treated TB cases were significantly higher than those in drug-susceptible isolates and new cases of TB (Table 3). The prevalence of \(pncA\) mutations in MDR-TB isolates (46.8%) collected from Zunyi, Guizhou Province, is lower than that in MDR-TB isolates collected from the neighboring Chongqing Province (57.9%, 77/133),\(^17\) but higher than those in MDR-TB isolates collected from Yunnan Province.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Distribution, mutant type and frequency of 64 \(pncA\) mutations detected in this study. Numbers are amino acid positions in the \(pncA\) coding region and numbers in brackets represent the number of isolates with the same mutation. Red symbol, MDR-TB; blue symbol, PDR-TB; and green symbol, Pan-S-TB.
Table 3 The Prevalence Of pncA Mutations In Drug-Resistant And Drug-Susceptible Isolates Of M. tuberculosis, And In Isolates From New And Previously Treated TB Cases

| First-Line Drugs And Treatment History | No. Of Isolates Tested | Isolates With pncA Mutation | No. % |
|---------------------------------------|------------------------|----------------------------|-------|
| **Rifampicin**                        |                        |                            |       |
| Susceptible                           | 189                    | 4                          | 2.1   |
| P value                               |                        |                            | p<0.01 χ²=54.0 |
| Resistant                             | 129                    | 60                         | 46.5  |
| **Isoniazid**                         |                        |                            |       |
| Susceptible                           | 148                    | 6                          | 4.1   |
| P value                               |                        |                            | p<0.01 χ²=26.4 |
| Resistant                             | 170                    | 58                         | 34.1  |
| **Ethambutol**                        |                        |                            |       |
| Susceptible                           | 250                    | 35                         | 14.0  |
| P value                               |                        |                            | p<0.01 χ²=26.4 |
| Resistant                             | 66                     | 28                         | 42.4  |
| **Streptomycin**                      |                        |                            |       |
| Susceptible                           | 208                    | 19                         | 9.1   |
| P value                               |                        |                            | p<0.01 χ²=43.8 |
| Resistant                             | 109                    | 44                         | 40.4  |
| **Multidrug-resistant TB**            |                        |                            |       |
| Pan-susceptible TB                    | 136                    | 3                          | 2.2   |
| P value                               |                        |                            | p<0.01 χ²=71.7 |
| New case                              | 188                    | 17                         | 9.0   |
| Previously treated case               | 130                    | 47                         | 36.2  |
| P value                               |                        |                            | p<0.01 χ²=35.14 |

(21.4%, 6/28)\(^{12}\) and Zhejiang Province (35.4%, 97/274)\(^{18}\) of China, supporting the suggestion that pncA mutations may differ from one geographic region to another based on studies from different regions.\(^{17}\)

To compare the prevalence of pncA mutations in MDR/RR, new, previously treated and total cases of TB in different settings around the world, we listed in Table 4 the published data from 32 countries in six WHO regions (Africa, Americas, Eastern Mediterranean, Europe, South-East Asia and Western Pacific). It was obvious that the prevalence of pncA mutations in M. tuberculosis isolates from different countries and different regions were quite different, consistent with the observation that levels of ZPA resistance varied substantially among different settings.\(^{7}\) For example, the prevalence of pncA mutations in MDR/RR (85%) and total cases of TB (57.9%) from George\(^{11}\) was much higher than that in MDR/RR cases from Turkey (25%)\(^{28}\) and in total cases of TB from Azerbaijan (12.6%),\(^{7}\) even though these three countries were located in the same European region (Table 4). The prevalence of pncA mutations among MDR-TB cases from 32 different countries in six WHO regions varied widely ranging from 21.4% in Yunnan Province of China\(^{12}\) and 39.5% in Pakistan\(^{7}\) to 81.3% in Belarus\(^{7}\) and 87.8% in Republic of Korea.\(^{38}\) In addition, the prevalence of pncA mutations among previously treated TB cases from six WHO regions also varied extensively ranging from 4.7% in South Africa,\(^{7}\) 8.9% in Pakistan\(^{7}\) and 13.8% in Bangladesh\(^{7}\) to 36.2% in Zunyi, China, 66.7% in Rwanda\(^{10}\) and 70.8% in George.\(^{11}\)

Sequencing results also showed that 52 different types of pncA mutations were detected from 64 clinical isolates of M. tuberculosis, mostly from MDR-TB isolates, but three from PDR-TB and three from Pan-S-TB isolates (Figure 1). The distribution, type, and frequency of 64 pncA mutations detected in this study were revealed in Figure 1. Five mutations were new and deposited in GenBank with accession numbers KR534845 (Ser67Stop), KR534846 (Tyr95del), KR534847 (Ser67Leu), KR534848 (Gly105FSC), and KR534849 (Gly113FSC). Based on a multicenter study, Miotto et al divided 280 genetic variants in pncA into four classes (i, ii, iii and iv), and class (i) mutations were very high confidence resistance ones that were found only in PZA-resistant strains.\(^{14}\) Among 52 mutation types (Table 2), 19 belonged in class (i), 2 belonged in class (ii) and 3 belonged in class (iii). Through comparison with the data published by the CRyPTIC consortium on whole-genome analyses of 10,209 isolates,\(^{40}\) we found that 25 mutations detected in MDR/PDR-TB isolates and two of three mutations detected in Pan-S isolates (R29C and H42D) fell in the “R” category, and one MDR (S65P) and one Pan-S isolates (Q141H) fell in the “S” category.

It was previously found in a systematic review of mutations reported in 61 studies that 79% of 2,760 PZA-resistant and 9% of 3,329 PZA-susceptible isolates had a mutation in the pncA gene,\(^{3}\) suggesting that M. tuberculosis clinical isolates with a mutation in the pncA gene be more likely resistant to PZA than those without a pncA mutation. The comparison results also indicated the existence of geographical diversity of prevalence of pncA mutations among M. tuberculosis clinical isolates depending on where they were collected. Therefore, we should consider geographical diversity as an important factor when we select screening of pncA mutations as a simple method for quick diagnosis of PZA resistance in M. tuberculosis isolates, and decide whether PZA should be included in the optimized treatment
Table 4 The Prevalence Of \textit{pncA} Mutations In \textit{M. tuberculosis} Isolates From Six Different WHO Regions And 32 Countries Of The World

| WHO Regions          | MDR/RR Cases | New Cases | PT Cases | Total Cases |
|----------------------|--------------|-----------|----------|-------------|
| Africa               |              |           |          |             |
| Burundi$^9$          | 65.7% (35)   | N/A       | N/A      | N/A         |
| Sub-Saharan Africa (12 countries)$^{10}$ | 54.3% (573) | 63.9% (72) | 49.0% (453) | 50.4% (125) |
| Cameroon$^{10}$      | 49.6% (133)  | 55.6% (9)  | 50% (116) |             |
| CAR$^{10}$           | 28.6% (35)   | N/A       | N/A      |             |
| DRC$^{10}$           | 73.7% (95)   | 75% (16)  | 68.7% (67) |             |
| Niger$^{10}$         | 39.6% (48)   | N/A       | 39.1% (46) | N/A         |
| Rwanda$^{10}$        | 67.3% (101)  | 69.0% (42) | 66.7% (36) | 67.9% (78)  |
| Senegal$^{10}$       | 33.3% (39)   | N/A       | 31.4% (35) | N/A         |
| South Africa         | 39.1% (39)   | 2.8% (648) | 4.7% (145) | 3.1% (877)  |
| Americas             |              |           |          |             |
| Brazil$^{19}$        | N/A          | N/A       | N/A      | 20.6% (97)  |
| Canada$^{20}$        | 88.5% (26)   | N/A       | N/A      | 37.6% (141) |
| Mexico$^{21}$        | N/A          | N/A       | N/A      | 26.7% (127) |
| Peru$^{22}$          | N/A          | N/A       | N/A      | 30.9% (68)  |
| Eastern Mediterranean|              |           |          |             |
| Iran$^{23}$          | N/A          | N/A       | N/A      | 27.3% (33)  |
| Pakistan$^{7}$       | 39.5% (103)  | 2.1% (1,299) | 8.9% (201) | 3.0% (1,500) |
| Europe               |              |           |          |             |
| Azerbaijan$^{7}$     | 59.9% (129)  | 10.2% (530) | 17.9% (218) | 12.6% (748) |
| Belarus$^{7}$        | 81.3% (98)   | 30.0% (144) | 69.9% (57) | 42.1% (201) |
| Georgia$^{11}$       | 85.0% (40)   | 48.5% (33) | 70.8% (24) | 57.9% (57)  |
| Germany$^{24}$       | 59.8% (82)   | N/A       | N/A      | N/A         |
| Kazakhstan$^{25}$    | 75% (48)     | N/A       | N/A      | 49.4% (77)  |
| Russia$^{26}$        | N/A          | N/A       | N/A      | 56.8% (44)  |
| Sweden$^{27}$        | N/A          | N/A       | N/A      | 45.1% (71)  |
| Turkey$^{28}$        | 25% (12)     | N/A       | N/A      | N/A         |
| Ukraine$^{29}$       | 73.6% (91)   | N/A       | N/A      | N/A         |
| South-East Asia      |              |           |          |             |
| Bangladesh$^{7}$     | 36.7% (63)   | 2.6% (751) | 13.8% (203) | 5.1% (955)  |
| India$^{20}$         | N/A          | N/A       | N/A      | 78.0% (50)  |
| Indonesia$^{31}$     | N/A          | N/A       | N/A      | 5.6% (322)  |
| Myanmar$^{32}$       | 60.0% (66)   | N/A       | N/A      | N/A         |
| Thailand$^{33}$      | 47% (100)    | N/A       | N/A      | 32.7% (150) |
| Western Pacific      |              |           |          |             |
| China                |              |           |          |             |
| Beijing$^{34}$       | 50% (142)    | N/A       | N/A      | N/A         |
| Chongqing$^{17}$     | 57.9% (133)  | N/A       | N/A      | 57.9% (133) |
| Jiangsu$^{35}$       | N/A          | N/A       | N/A      | 15.0% (254) |
| Shanghai$^{36}$      | N/A          | N/A       | N/A      | 41.0% (432) |
| Sichuan$^{35}$       | N/A          | N/A       | N/A      | 15.2% (408) |
| Southern China$^{36}$| 31.0% (129)  | N/A       | N/A      | 15.5% (878) |
| Yunnan$^{12}$        | 21.4% (28)   | N/A       | N/A      | 11.1% (54)  |
| Zhejiang$^{35}$      | 35.4% (274)  | N/A       | N/A      | 16.7% (216) |
| Zunyi                 | 46.8% (124)  | 9.0% (188) | 36.2% (130) | 20.1% (318) |
| Japan$^{37}$         | 66.7% (36)   | N/A       | N/A      | N/A         |

(Continued)
regimens for TB patients, particularly those from countries with high prevalence of pncA mutations in MDR/RR and previously treated TB cases.

Limitations of this study included that we only collected smear-positive specimens to make sure the sputum specimens to be positive of bacteria at the time of collection and to grow enough bacteria for DST and DNA extraction. If we had also collected smear-negative specimens for this study, the overall prevalence of pncA mutations would have been lower but should not affect the conclusion of this study. Another limitation was that culture-based phenotypic testing for PZA was not performed on clinical isolates of M. tuberculosis due to its technical difficulties.

Conclusion
Results from this study show that high prevalence of pncA mutations in clinical isolates of M. tuberculosis from Zunyi, Guizhou Province of China, are correlated with resistance to each of the four first-line anti-TB drugs, MDR-TB and previously treated TB cases.

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Author Contributions
ZC, YL, MX and ZP carried out the experiments. All authors contributed to data analysis, drafting and revising the manuscript, agreed to be accountable for all aspects of the work, and approved the final version of the manuscript.

Disclosure
HZ is employed by and has shares in Z-BioMed, Inc., which is involved in infectious disease research. The authors report no other conflicts of interest in this work.

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