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ORIGINAL RESEARCH

Detection of Novel Gene Mutations Associated with Pyrazinamide Resistance in Multidrug-Resistant *Mycobacterium tuberculosis* Clinical Isolates in Southern China

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Objective: Pyrazinamide (PZA) is a cornerstone of modern tuberculosis regimens. This study aimed to investigate the performance of genotypic testing of *pncA*$^{+}$ upstream region, *rpsA, panD, Rv2783c,* and *clpC1* genes to add insights for more accurate molecular diagnosis of PZA-resistant (R) *Mycobacterium tuberculosis.*

Methods: Drug susceptibility testing, sequencing analysis of PZA-related genes including the entire operon of *pncA* (*Rv2044c-pncA-Rv2042c*) and PZase assay were performed for 448 *M. tuberculosis* clinical isolates.

Results: Our data showed that among 448 *M. tuberculosis* clinical isolates, 113 were MDR, 195 pre-XDR and 70 XDR TB, while the remaining 70 strains had other combinations of drug-resistance. A total of 60.04% (269/448) *M. tuberculosis* clinical isolates were resistant to PZA, of which 78/113 were MDR, 119/195 pre-XDR and 29/70 XDR TB strains. PZA^{R} isolates have predominance (83.3%) of Beijing genotype. Genotypic characterization of *Rv2044c-pncA-Rv2042c* revealed novel nonsynonymous mutations in *Rv2044c* with negative PZase activity which led to confer PZA^{R}. Compared with phenotypic data, 84.38% (227/269) PZA^{R} strains with mutations in *pncA*$^{+}$ upstream region exhibited 83.64% sensitivity but the combined evaluation of the mutations in *rpsA* 2.60% (7/269), *panD* 1.48% (4/269), *Rv2783c* 1.11% (3/269) and *Rv2044c* 0.74% (2/269) increased the sensitivity to 89.59%. Fifty-seven novel mutations were identified in this study. Interestingly, a frameshift deletion (C−114del) in upstream of *pncA*^{wt} nullified the effect of A−11G mutation and induced positive PZase activity, divergent from five PZase negative A−11G PZA^{R} mutants. Twenty-six PZA^{R} strains having wild-type-sequenced genes with positive or negative PZase suggest the existence of unknown resistance mechanisms.

Conclusion: Our study revealed that PZA^{R} rate in MDR and pre-XDR TB was markedly higher in southern China. The concomitant evaluation of *pncA*$^{+}$ UFR, *rpsA, panD, Rv2783c,* and *Rv2044c* provides more dependable genotypic results of PZA resistance. Fifty-seven novel mutations/indels in this study may play a vital role as diagnostic markers. The upstream region of *pncA* and PZase regulation are valuable to explore the unknown mechanism of PZA-resistance.

Keywords: tuberculosis, pyrazinamidase, drug resistance, molecular diagnosis, novel mutations, frameshift deletion

Introduction

Pyrazinamide (PZA), an analog of nicotinamide, is a key component of current and new anti-tuberculosis (TB) regimens for treatment of both drug-susceptible and multidrug-resistant (MDR) TB, because it plays a critical role in shortening the TB

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therapy from 9 to 12 months to 6 months.[1] PZA has a unique sterilizing activity against *Mycobacterium tuberculosis* persisters that are not killed effectively by other anti-TB drugs, but its mechanism of action is complex and not well understood yet.[2]

PZA is a prodrug that is converted to its active form, pyrazinoic acid (POA), by an enzyme, pyrazinamidase (PZase), encoded by *pncA* gene.[3] Averagely, 70% to 90% of PZA resistance (R) incidences are due to mutations in the *pncA* gene.[4,5] In recent studies, RpsA which encodes the ribosomal protein S1, involved in translation and trans-translation, has been reported as a target of PZA.[6] The mutations in *rpsA* seem to have a minor role in PZA^R in the clinical isolates having no mutation in *pncA* gene.[7] Besides, another gene *panD* encoding aspartate decarboxylase involved in beta-alanine and pantothenate synthesis was recently identified, whose mutations were also associated with PZA^R in *M. tuberculosis* strains lacking *pncA* and *rpsA* mutations.[8]

In our recent study, we discovered Rv2783 as a new potential target of POA in *M. tuberculosis*.[9] Rv2783, a bifunctional enzyme which was proved to be able to synthesize and hydrolyze (p)ppGpp and to synthesize and hydrolyze ssDNA and ssRNA without any template.[9] In addition to the above genes, ClpC1 was reported as another new target of PZA which acts as an unfoldase in concert with the proteases ClpP1 and ClpP2 of the caseinolytic protease complex but the mutations in the coding region of ClpC1 were associated with PZA^R.[10]

The Wayne assay exhibits the association between PZase activity and genetic mutations in PZA-associated genes. Mutations in *pncA* gene are considered to be responsible for the failure of key-lock recognition mechanism between the enzyme and its substrate. Similarly, when mutation occurs in the upstream flanking region (UFR) of *pncA*, the RNA polymerase may not be able to transcribe it. Thus, there is no PZase activity and PZA cannot be converted into POA.[11] *pncA* (*Rv2043c*) is co-transcribed as a polycistron (*Rv2044c-pncA-Rv2042c*) with its upstream gene *Rv2044c* and downstream gene *Rv2042c*.[12] However, apart from frequently observed mutations at position −11 in the putative regulatory region of *pncA*, no reports showed yet that mutations in the instant upstream gene of wild-type (wt) $pncA^{wt}$ may develop PZA^R in *M. tuberculosis*.

The prior studies described the correlation between PZA^R and *pncA* mutations. Whereas no study has been conducted so far for simultaneous characterization of the mutations in $pncA^+$ UFR, *rpsA, panD, Rv2783c,* and *clpC1* for the detection of PZA^R in MDR, pre-XDR (MDR with additional resistant to fluoroquinolone or a second-line injectable drug, e.g. kanamycin, amikacin) and XDR (MDR in addition with resistant to both fluoroquinolones and second-line injectable drugs) *M. tuberculosis* clinical isolates. The currently available phenotypic PZA susceptibility testing methods are complicated and deceptive because of frequent false-resistance and false-susceptible results.[13] Genotypic characterization of PZA-related genes has been endorsed to overcome the shortcomings of phenotypic susceptibility testing methods.[14]

In this study, to evaluate the performance of genotypic testing method of PZA-associated genes for detecting PZA^R strains, we performed the phenotypic and genotypic characterization of 448 *M. tuberculosis* clinical isolates from southern China. Considering the observation that UFR of *pncA* has an indispensable role in PZase regulation as well as PZA^R,[11,12] we have additionally evaluated the complete operon of *pncA* (*Rv2044c-pncA-Rv2042c*) to identify the possible role of surrounding genes of *pncA* in PZA^R *M. tuberculosis*.

Materials and Methods

Ethical Approval

The current study was approved by the Ethics Committee of Guangzhou Chest Hospital (GZXK-2016-015) in accordance with the WHO-approved guidelines.

Collection of *M. tuberculosis* Clinical Isolates

In this study, 448 drug-resistant *M. tuberculosis* clinical isolates (resistant ≥ one anti-TB drug) were collected during the period from December 2016 to November 2018 from TB patients at Guangzhou Chest Hospital, the biggest TB-specialized hospital in southern China. Ziehl–Neelsen staining and commercial MPB64 monoclonal antibody assay (GENESIS, Hangzhou, China) were performed to confirm the *M. tuberculosis* species.[15]

Drug Susceptibility Testing

Drug susceptibility testing (DST) of 448 *M. tuberculosis* isolates was first assessed by Mycobacterial Growth Indicator Tubes (MGIT) 960 (Becton Dickinson, Franklin Lakes, NJ, USA). The critical concentrations (μg/mL) for DST were consistent with WHO recommendations; isoniazid (INH; 0.1), rifampicin (RIF; 1.0), ethambutol (EMB; 5.0), PZA (100), streptomycin (STR; 1.0), levofloxacin (LVX; 1.0), moxifloxacin (MXF; 0.25) and amikacin (AMK; 1.0).[16] In order to measure the extent of

phenotypic PZA^R by the gold standard MGIT 960 system, the higher concentrations of PZA (300 and 900 µg/mL) were tested particularly for those PZA^R (100 µg/mL) strains which showed inconsistent phenotypic and genotypic results. The DST results were also verified via indirect proportion method on Löwenstein–Jensen medium using the recommended concentrations (µg/mL); INH (0.2), RIF (40.0), EMB (2.0), STR (4.0), LVX (2.0), MXF (1.0) and AMK (30.0).[16] The growth on a control medium (drug-free medium) was compared with the growth on drug-containing medium and the resistance was determined when 1% or more growth was noticed at the critical concentration of drug in the medium.[16]

PZase Activity Assay

PZase activity assay was performed with some modifications in a Wayne test[8] to determine the PZase regulation. Briefly, 3 to 4 pure and freshly grown *M. tuberculosis* colonies on LJ medium were scraped off and transferred into 1 mL Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC) containing PZA (100 and 200 µg/mL) in 1.5 mL Eppendorf tubes. The colonies were incubated at 37°C in a shaker for 3, 5, and 7 days. Later, 15 µL of 2% Fe^{2+} was added into the bacterial cells and incubated at 4°C for 2–4 hrs for color appearance. *M. tuberculosis* H37Rv^{wt} (pyrazinamide-susceptible; PZA^S) and *M. bovis* Bacillus Calmette-Guérin (BCG) Tice (PZA^R) were used as positive and negative controls, respectively.

PZA-Associated Genes Amplification and Sequencing

Genomic DNA was extracted from freshly grown *M. tuberculosis* cultures using MagMAX Total Nucleic Acid Isolation Kit (Ambion, Life Technologies, NY, USA) according to the manufacturer's instructions. All PZA-associated genes (*pncA, rpsA, panD, Rv2783c* and *clpC1*) including their 5′ upstream (≤ − 200 bp) to 3′ downstream (≤ + 200 bp) regions were amplified in all *M. tuberculosis* isolates using the newly designed primers in this study (Table 1). The complete operon of *pncA* (*Rv2044c-pncA-Rv2042c*) was additionally analyzed in PZA^R strains. PCR products were examined on agarose gels, purified by PCR purification kit (Qiagen, Hilden, Germany) and sequenced at the BGI (Guangzhou, China). The DNA sequences were compared with the reference sequence of *M. tuberculosis* H37Rv (Accession number: NC_000962.3) using the software BioEdit version 7.2.6.1.

Detection of Beijing and Non-Beijing Genotypes

Multiplex PCR method was used to distinguish Beijing and non-Beijing genotypes as previously described[17] that region spanning genes *Rv2816* to *Rv2819* including part of *Rv2820* is missing in Beijing genotype of *M. tuberculosis* strain. A set of primers BJ-F: 5′-ACCGAGCTGATCAAACCCG -3′ and BJ-R: 5′-ATGGCACGGCCGACCTGAATGAACC -3′ amplified the 239 bp PCR product containing region-specific part of *Rv2819* and part of *Rv2820* in Beijing genotypes. Whereas another pair of primers NBJ-F: 5′-GATCGCTTGTTCTCAGTGCAG-3′ and NBJ-R: 5′-CGAAGGAGTACCACGTGGAG-3′ is used to detect non-Beijing genotypes by amplification of 539 bp PCR fragment from *Rv2819* gene. The PCR products were observed on agarose gels.

Statistical Analysis

Phenotypic PZA susceptibility testing and the PZase assay were used as references. Associations among multiple aspects between PZA^S and PZA^R strains were analyzed with Pearson Chi-square test. A paired Chi-square test was used via MEDCALC® statistical software (https:/www.medcalc.org/calc/diagnostic_test.php) to measure the sensitivity, specificity, odds ratio (OR), 95% confidence interval (CI) and accuracy of PZA genotypic susceptibility testing method. *P*-value of <0.05 was considered statistically significant.

Results

Demographic Characteristics

Of the 448 *M. tuberculosis* isolates, 71.20% (319/448) were from male patients and 28.79% (129/448) were from female patients. Age range was 15 to 89 years with a mean age of ~41.2 years. Majority of TB patients 62.5% (280/448) examined in this study were previously treated cases and 69.86% (313/448) TB patients enrolled in the current study was migrant population.

Drug Susceptibility Profiles of *M. tuberculosis* Clinical Isolates

Among the 448 *M. tuberculosis* clinical isolates, 113 were identified as MDR, 195 pre-XDR and 70 XDR TB, while the remaining 70 strains had a random pattern of drug resistance. Total 60.04% (269/448) *M. tuberculosis* clinical isolates assessed by gold standard MGIT 960 system were PZA^R in this study which were categorized as 69.02% (78/113) MDR,

Table 1 Primers and PCR Products of PZA-Associated Genes with Brief Explanatory Notes

Gene	Protein	Functional Activity	Primer Name	Oligonucleotide Sequence (5′→3′)	Product Size ~ (−200 to + 200)
pncA (*Rv2043c*)	Pyrazinamidase/ nicotinamidase (PZase)	Convert amide into acid (PZA into POA)	pncA FpncA R	TCGCTCACTACATCACCGGC TCGTAGAAGCGGCCGATGGC	892 bp
rpsA (Rv1630)	30S ribosomal protein S1 (RpsA)	Trans-translation	rpsA F rpsA R	ACTGAGTGCCGAGCGTGCATC ACCGAACGCGTCGACCAGCG	1800 bp
panD (*Rv3601c*)	Aspartate alpha-decarboxylase (PanD)	Pantothenate biosynthesis	panD F panD R	TCGACTACCTGGAGCTGCGC TCGATCGTCAGTGCCAGTTC	755 bp
Rv2783c	Bifunctional protein polyribonucleotide Nucleotidyltransferase (GpsI, Pnpase) and synthesize and hydrolyze (p) ppGpp	Synthesis/ degradation of ssDNA/ssRNA and (p)ppGpp	gpsI F gpsI R	ATTCAGACCTTTTCTCCTGGG GTCGACTTGAACAGCAAATG	2547 bp
clpC1 (*Rv3596c*)	ATP-dependent protease ATP-binding subunit (ClpC1)	Hydrolyses proteins in presence of ATP	clpC1 F clpC1 R	ACGCTTGGGTGGTTTTCTCGTT ACAAACCGACGTCAGCAGAGT	2816 bp
**Rv2044c-pncA-Rv2042c*	Conserved hypothetical protein- PZase- Conserved protein		EO pncA F EO pncA R	GTGCCGCATCGAGTTCGATCCGCA GATATCGGGATAGCGCCGCTGGA	2070 bp

Notes: Primers were based on the *M. tuberculosis* H37Rv genome sequence (Accession No.: NC_000962; Version: NC_000962.3). *Entire operon of *pncA*.

61.02% (119/195) pre-XDR and 41.42% (29/70) XDR *M. tuberculosis* which collectively covered 59.8% (226/378) of the total (M/pre-X/X-DR) TB strains (Table 2).

Genotypes of PZA-Resistant Isolates

Genotyping by multiplex PCR-based method demonstrated that overall 80.58% (361/448) belonged to Beijing genotype and the other 19.42% (87/448) to non-Beijing genotype in total studied isolates. The distribution of these total Beijing and non-Beijing genotypes in PZA^R and PZA^S strains is given in Table 3. PZA^R isolates predominantly (83.3%; 224/269) belonged to the Beijing genotype, whereas, 16.7% (45/269) PZA^R isolates were from non-Beijing genotype family of *M. tuberculosis* strains.

Association Between Mutations in *pncA* + UFR and PZase Enzyme Activity

Among the 84.38% (227/269) PZA^R strains detected with *pncA* + UFR mutations (Table S1), 23.78% (54/227) exhibited insertions/deletions (indels) in *pncA* coding region, 5.72% (13/227) had stop codon mutations, 59.03% (134/227) carried single nonsynonymous mutation, 0.44% (1/227) had multiple mutations, 0.88% (2/227) showed synonymous mutation and 5.28% (12/227) had mutations both in

Table 2 Prevalence of MDR, Pre-XDR and XDR TB in PZA^R Strains

Drug Resistance Profile	Total Isolates	PZA^R Isolates	Proportion (%)	PZA^S Isolates	Proportion (%)
MDR TB	113	78	69.02*	35	30.97
Pre-XDR TB	195	119	61.02*	76	38.97*
XDR TB	70	29	41.42	41	58.57*
Varied pattern#	70	43	61.42	27	38.57
Total	448	269	60.04	179	39.95

Notes: *Indicates significantly higher rate than XDR TB in PZA^R isolates while in PZA^S isolates significantly higher rate than MDR TB measured by Chi-square test. **#** shows the number of drug-resistant strains with several distinct combinations of resistance against tested anti-TB drugs. MDR TB: *M. tuberculosis* strain resistant to at least INH and RIF. Pre-XDR TB: MDR strain additionally resistant to either a fluoroquinolone (FQ) or a second-line injectable drug but not both at the same time. XDR TB: MDR TB along with resistant to any fluoroquinolone (FQ) and at least one injectable second-line drug (e.g. amikacin, kanamycin, etc.) simultaneously.

Table 3 Distribution of Beijing and Non-Beijing Genotypes

Genotype	Total Isolates		PZA^R Isolates n =269	Proportion (%)	PZA^S Isolates n = 179	Proportion (%)
	n = 448	Proportion (%)				
Beijing genotype	361	80.58	224	83.3*	137	76.5
Non-Beijing genotype	87	19.42	45	16.7	42	23.5

Note: * Beijing genotype is significantly higher in PZA^R isolates measured by Chi-square test.

pncA and its UFR. The amino acid substitutions/indels in *pncA* were scattered over the gene including the nonsynonymous mutations near the active site or metal-binding site. However, 4.84% (11/227) PZA^R strains had mutations/indels only in the UFR of *pncA*^{wt} with frequently existed substitutions at −11 position. The PZase assay revealed that 3.96% (9/227) PZA^R strains had positive or weakly positive PZase activity, though they had indels or synonymous mutation in *pncA* gene or its UFR while the remaining 96.03% (218/227) were PZase negative (Table 4).

In addition, among the substitutions located at −11 position, five A−11G PZA^R mutants were detected with negative PZase activity but a very interesting phenomenon was identified in this study that one A−11G PZA^R mutant having a deletion of C nucleotide at position −114 in the UFR of *pncA* showed positive PZase activity, which suggests that C−114del possibly altered the effect of A−11G mutation and induced the normal regulation of PZase (PZase positive) in this PZA^R strain. On the whole, 21.14% (48/227) new nonsynonymous mutations/indels were found in *pncA* and its UFR in this study.

Mutations in *rpsA, panD, Rv2783c* and *clpC1*

In this study, 2.60% (7/269) PZA^R strains carried nonsynonymous mutations (Thr29Met; Gln162Arg; Ala412Val) in RpsA (Table S2). Two of them (Gln162Arg, Ala412Val) were identified first time in this study. A synonymous mutation (Arg212Arg) in RpsA was frequently observed in both PZA^S and PZA^R strains. Further, we found 1.48% (4/269) PZA^R strains with Leu132Pro and Pro134Ser mutations in the C-terminus of PanD which possibly alter the protein structure and cause resistance. The mutation Leu132Pro is recognized as a novel mutation in PanD. It is interesting to note that, a new nonsynonymous mutation (Ser149Pro) was detected in *Rv2783c* of 1.11% (3/269) PZA^R strains, whereas no mutation occurred in *clpC1* gene or its promoter region.

Characterization of *Rv2044c-pncA-Rv2042c* in *pncA*^{wt} PZA^R Strains

Most importantly, 10.40% (28/269) PZA^R strains were found without mutation in the above-sequenced genes. The phenotypic resistance of these strains were reconfirmed by using higher concentration (300 and 900 µg/mL) of PZA and the genotypic assessment of entire operon of *pncA* (*Rv2044c-pncA-Rv2042c*) was performed. It is worth noting that among the total PZA^R strains, 0.74% (2/269) PZA^R strains were identified with nonsynonymous mutations (Tyr70His, Ile71Asn, Trp80Gly) and (Trp80Leu, Ala82Asp) in Rv2044 (the instant upstream gene of *pncA*) and produced negative PZase activity (Figure 1). Interestingly, 9.7% (26/269) of the

Table 4 Correlation of Genotypic Susceptibility Testing and PZase Activity Assay

PZase Activity	PZA^R Isolates								Total
	No Mutation	Mutants							
	WT Genes	*pncA*		*Rv2044c*	*rpsA*	*panD*	*Rv2783c*	*clpC1*	
		UFR	*pncA*						
No. of strains	26	11	216	2	7	4	3	–	269
PZase negative	9	7	211	2	–	–	–	–	229
PZase positive	17	4	5	–	7	4	3	–	40

Note: PZA^S strains (179/448) were observed with positive PZase activity.
Abbreviations: PZase, pyrazinamidase; UFR, upstream flanking region of *pncA*.

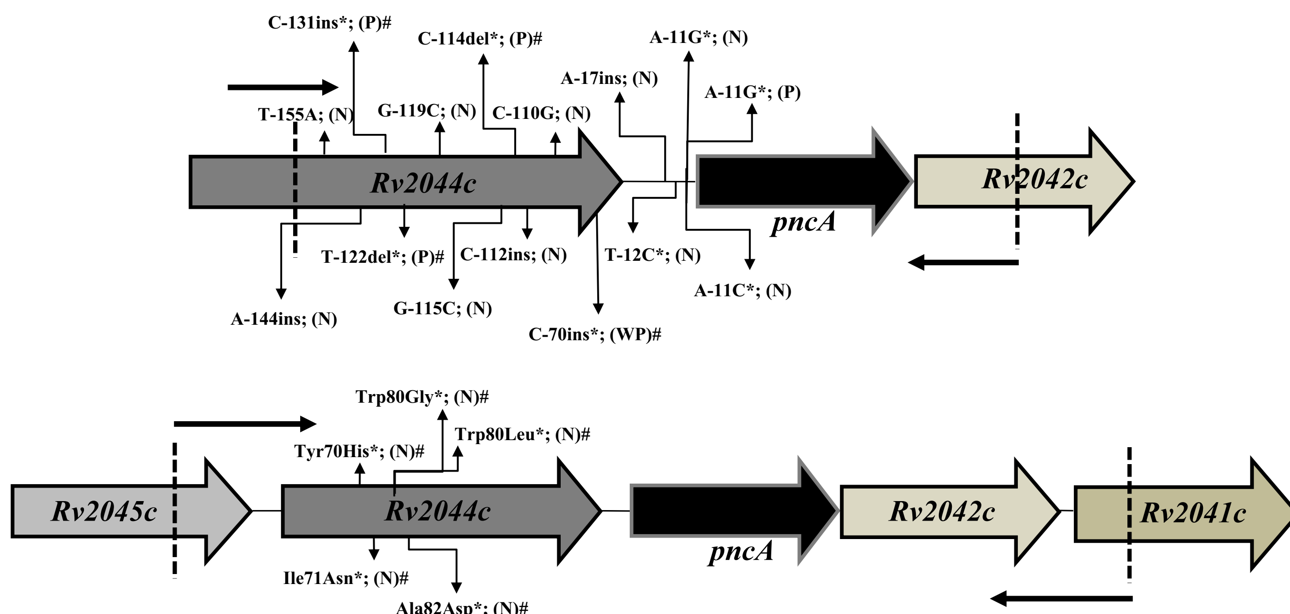

Figure 1 Depiction of mutations in *Rv2044c-pncA-Rv2042c* operon in PZA^R strains. *Indicates the mutations exist only in *Rv2044c* or upstream flanking region of *pncA*^{wt} in PZA^R *M. tuberculosis* strains. The remaining mutations in operon exist along with the mutation(s) in *pncA* coding region. # indicates the novel mutations. Small arrows near the dotted lines direct the forward and reverse primers used for amplification of required fragments. PZase activity is shown by letters; N (negative), P (positive), WP (weakly positive).

total PZA^R strains were discovered without any mutation in all known PZA-associated genes. Among these 26 PZA^R strains, 34.61% (9/26) produced negative PZase activity and 65.38% (17/26) PZA^R strains had positive PZase activity (Table 4) in PZase assay. The genotypic reassessment also confirmed no mutation in all sequenced genes in these PZA^R strains. Overall, 23.45% (57/243) novel mutations/indels were identified in *pncA* + UFR, *rpsA, panD, Rv2783c* and *Rv2044c* genes among the total 90.3% (243/269) genetic mutations in PZA^R strains.

DNA Sequencing Analysis of Phenotypic PZA^S Strains

Of the total 39.95% (179/448) PZA^S strains, 1.67% (3/179) harbored synonymous mutations, 0.55% (1/179) insertion and 3.35% (6/179) were noticed with single nonsynonymous mutations only in *pncA* of phenotypic PZA^S strains with positive PZase activity (Table S3), while 94.41% (169/179) PZA^S strains were found with wild-type *pncA* and other sequenced genes along with positive PZase activity.

Prediction of PZA^R Isolates Based on DNA Sequencing

To examine the performance of DNA sequencing method for detection of PZA^R isolates, the genotypic and phenotypic results of 448 *M. tuberculosis* isolates were compared. Using the phenotypic results as a reference, the sequencing method exhibited 75.09% sensitivity and 96.09% specificity for the mutations only in the *pncA* coding region, but the genotypic characterization of *pncA* accompanied by its UFR improved the sensitivity to 83.64% while specificity remained unchanged. The individual sequencing of *rpsA, panD, Rv2783c,* and *Rv2044c* showed low sensitivities, indicating minor diagnostic role of their own. However, the combined evaluation of mutations in *pncA* + UFR, *rpsA, panD, Rv2783c,* and *Rv2044c* genes increased the sensitivity from 75.09% to 89.59% with 92.19% accuracy and 96.09% specificity (Table 5).

Evaluation of Association of PZase Assay with MGIT 960 and DNA Sequencing

Phenotypically PZA^R *M. tuberculosis* strains assessed by MGIT 960 usually show PZase negative activity (PZase -) because of mutation in *pncA* gene which disrupts the PZase regulation. Whereas PZA^R *M. tuberculosis* strains without *pncA* mutation possess positive PZase activity (PZase +). The correlation between PZase activity assay and MGIT 960 showed the sensitivity (85.13%, [95% CI: 80.31–89.16]) and specificity (100%, [95% CI: 97.96–100]) with the accuracy of (91.07%, [95% CI: 88.04–93.54]) due to the high number (269/448) of PZA^R strains. Besides, the

Table 5 Evaluation of Sequencing Method and Phenotypic Susceptibility Testing for Detection of PZA Resistance

Locus	PZA^R Isolates n=269		PZA^S Isolates n=179		% Sensitivity (95% CI)	% Specificity (95% CI)	% Accuracy (95% of CI)
	Nonsynonymous Mutation & Indel	Wildtype or Synonymous Mutation	Nonsynonymous Mutation & Indel	Wildtype or Synonymous Mutation			
UFR	11 (4.08)	258 (95.91)	0 (0.0)	179 (100)	4.09 (2.06–7.20)	100 (97.96–100.0)	42.41 (37.79–47.14)
pncA + UFR	12 (4.46)	257 (95.53)	0 (0.0)	179 (100)	4.46 (2.33–7.66)	100 (97.96–100.0)	42.63 (38.0–47.36)
pncA^c	202 (75.09)	67 (24.90)	7 (3.91)	172 (96.08)	75.09 (69.48–80.14)	96.09 (92.11–98.41)	83.48 (79.71–86.80)
*pncA**	225 (83.64)	44 (16.35)	7 (3.91)	172 (96.08)	83.64 (78.67–87.86)	96.09 (92.11–98.41)	88.62 (85.30–91.41)
Rv2044c	2 (0.74)	267 (99.25)	0	179 (100)	0.74 (0.09–2.66)	100 (97.96–100.0)	40.40 (35.82–45.11)
rpsA	7 (2.60)	262 (97.39)	0	179 (100)	2.60 (1.05–5.29)	100 (97.96–100.0)	41.52 (36.91–46.24)
panD	4 (1.48)	265 (98.51)	0	179 (100)	1.49 (0.41–3.76)	100 (97.96–100.0)	40.85 (36.26–45.56)
Rv2783c	3 (1.11)	266 (98.88)	0	179 (100)	1.12 (0.23–3.22)	100 (97.96–100.0)	40.62 (36.04–45.33)
clpC1	0 (0.0)	269 (100)	0	179 (100)	0 (0–1.36)	100 (97.96–100)	39.96 (35.39–44.66)
All*	241 (89.59%)	28 (10.40%)	7 (3.91)	172 (96.08)	89.59 (85.31–92.97)	96.09 (92.11–98.41)	92.19 (89.30–94.50)

Notes: UFR = represents the strains with mutations only in UFR of *pncA*. *pncA* + UFR = represents the strains having mutations both in UFR and *pncA*. *pncA*^{C} = represents the strains containing the mutations only in the coding region of *pncA*. *pncA** = represents the combined evaluation of strains covering the entire coding region of *pncA* and UFR together. All*= Combined evaluation of all genes.
Abbreviations: PZA^R, PZA-resistant; PZA^S, PZA-susceptible; UFR, Upstream flanking region; Indel, Insertion, deletion; 95% CI, 95% Confidence interval.

association between genotypic testing and PZase assay exhibited the sensitivity (81.78%, [95% CI: 76.64–86.21]) and specificity (96.09%, [95% CI: 92.11–98.41]) with the accuracy of (87.50%, [95% CI: 84.08–90.42]) because of high incidence of resistance-conferring *pncA*$^+$ UFR mutations.

Discussion

This study shows the prevalence rates of PZA^R in MDR (69%) and pre-XDR (61%) TB in southern China were higher than the MDR TB observed in South Africa (52.1%), Japan (52.8%) and Thailand (49.0%),[18,19,20] but in line with another recently published study.[21] Beijing genotype was the most dominant genotype 80.58% (361/448) observed in southern China, similar to the studies from Beijing[22] and Zhejiang.[23] PZA has an imperative role in current and new anti-TB regimens including the promising Pa-824 + Moxifloxacin + PZA, bedaquiline + PZA[24] and clofazimine + PZA containing regimens.[25] Our study comprehensively investigates the genetic mutations in PZA-related genes (*pncA, rpsA, panD, Rv2783c*, and *clpC1*) in addition with the complete operon of *pncA* (*Rv2044c-pncA-Rv2042c*) in a wide-range of *M. tuberculosis* clinical isolates.

The detection of 84.38% (227/269) *pncA* + UFR mutations in PZA^R strains in this study was markedly higher than previous reports from Thailand (75.0%),[19] Brazil (45.7%)[26] and other parts of China (78.0%).[23] Though mutations in *pncA* scattered all over the gene and its UFR but a certain frequency of *pncA* mutations has been observed at amino acid residues 3 to 12, 46 to 62, 67 to 85, 94 to 103, and 132 to 142 which were the areas close to the PZase active sites (Asp8, Lys96, and Val139) or metal ion binding sites (Asp49, His51, His57, and His71).[23] Our findings are coherent with the previous studies.[20,23] So, the absence of "hot spot" regions make it quite challenging to develop rapid diagnostic assays for detection of PZA^R isolates based on indel or nonsynonymous mutations in the *pncA*.[13,27]

The *rpsA* gene has been ambiguously discussed in the literature as both supportive and opposing studies have been

published regarding the role of RpsA in PZA^R strains.[12,28,29] However, two novel mutations (Gln162Arg; Ala412Val) are identified in RpsA of PZA^R isolates in this study. Moreover, mutations in *panD* have also been associated with PZA^R in clinical isolates lacking mutations in *pncA* and *rpsA*.[8] The C-terminus of PanD protein spanning amino acid residues 114–139, where all the PanD mutations are usually mapped may affect the binding of the active form of PZA (POA) without abolishing PanD's enzymatic activity.[30] We discerned (Leu132Pro and Pro134Ser) mutations in the C-terminus of PanD in PZA^R isolates. Leu132Pro is recognized as a novel mutation in our study. Though another study reported (Leu132Arg) and (Leu136Arg) mutations in PanD[31] but these mutations were different from our findings regarding the amino acid substitutions.

Furthermore, Rv2783 plays an important role in the general homeostasis of (p)ppGpp during dormancy; however, POA inhibits its function in wild-type strain, but the Asp67Asn mutation helps in circumvention of POA effects.[9] In this study, we identified three PZA^R strains with positive PZase activities bearing one novel nonsynonymous mutation (Ser149Pro) in Rv2783, which supports that Rv2783 is a potential target of POA.[9] Likewise, *clpC1* (*Rv3596c*) involved in protein degradation by assembling a protease complex through ClpP1 and ClpP2, was proposed to be a new target of PZA and the mutations (Gly99Asp, Lys209Glu) in N-terminal and D1 domain have been interrelated with PZA^R strains.[10,27] However, there was no any mutation in *clpC1*of *M. tuberculosis* clinical isolates in this study. This degradative protease is essential for the viability, survival, and virulence of *M. tuberculosis*. Therefore, the *M. tuberculosis* strains containing mutations in ClpC1 may not be able to survive well in the host, so it is hard to get such mutants in clinical isolates.[7,32]

On the other hand, 10.40% (28/269) PZA^R strains were observed without any mutation in *pncA, rpsA, panD, Rv2783c,* and *clpC1* genes. However, to determine the significant role of UFR of *pncA* and its surrounding genes in PZase regulation,[11,12] we sequenced the complete operon of *pncA* (*Rv2044c*-*pncA*-*Rv2042c*) in these PZA^R strains. Notably, two of these PZA^R strains were detected with novel missense mutations "Tyr70His, Ile71Asn, Trp80Gly" and "Trp80Leu, Ala82Asp" in Rv2044 first time in this study. Interestingly, these strains exhibited the negative PZase activity in PZase assay which was likely to be the effect of these mutations. However, in-depth impact of these mutations on PZase regulation needs to be investigated in imminent studies. This observation is also supported by another recent study where an important frameshift deletion was identified in *Rv2044c* that interrupted the stop codon and led to its fusion with *pncA* which engendered the addition of a novel domain of unknown function (DUF2784) to the PZase enzyme.[33] Overall, 23.45% (57/243) novel mutations/indels were detected in PZA^R strains in our study.

PZA^S strains with missense mutations/insertion in *pncA* were reconfirmed by phenotypic and genotypic assays. The mutation (T92G→Ile31Ser) has been previously described in both PZA^S and PZA^R isolates with similar PZase characteristics,[19] whereas Thr47Ala was also reported without its involvement in PZA^R.[34] The mutations in susceptible strains were unable to encounter the threshold of resistance.[13] No nonsynonymous mutations/indels were identified in the genes other than *pncA* in PZA^S strains.

Compared to the phenotypic data, the sensitivity for detecting PZA^R by DNA sequencing of *pncA* gene along with its UFR was 83.64% with 96.09% specificity which was consistent with the data from South Korea (84.6%)[35] and the United States (84.6%),[36] only a little higher than the studies from northern China (77.97%),[23] Thailand (75%)[19] and Sierra Leone (70%)[37] but lower than the findings from Netherlands (96.8%).[38] Moreover, the combined assessment of mutations in *pncA* + UFR, *rpsA, panD, Rv2783c,* and *Rv2044c* increased the sensitivity up to 89.59% which strengthens their share for detection of PZA^R *M. tuberculosis* clinical isolates.

In agreement with the several studies, the current study demonstrated that mutations in *pncA* gene turns the regular enzyme activity into PZase negative; however, nine PZA^R strains with synonymous mutations/indels in the *pncA* or its UFR in our study retained the positive/weakly positive PZase activity and may have the involvement of enigmatic mechanism(s) of resistance against PZA. The PZase activity assay in association with MGIT 960 susceptibility testing showed 85.13% sensitivity. In the same way, the correlation between PZase assay and genotypic testing presented the sensitivity of 81.78% and 96.09% specificity in our study, which is significantly higher than previously published study (68.6%) and (45.7%)[26] but slightly lower than another report 88.2% and 88.8%,[39] respectively. Similar to previous reports, the mutations in *rpsA, panD* and *Rv2783c* were not found to be associated with loss of PZase activity in PZA^R strains lacking *pncA* mutation.[6,8,9,26,30]

In contrast, the substitution at position −11 of *pncA* usually possess negative PZase activity in PZA^R strains.[40] While we identified one A−11G PZA^R mutant having a deletion of C nucleotide at position −114 in the UFR of *pncA* showed PZase positive, that was completely divergent from five other PZase negative A−11G PZA^R mutants, which proposes that C−114del might turn the impact of A−11G mutation and produced positive PZase activity in this PZA^R strain. This needs to be further studied in the forthcoming studies. Moreover, the consistent results of phenotypic and genotypic reassessment of 34.61% (9/26) PZase negative and 65.38% (17/26) PZase positive PZA^R strains without any known mutation in all reported PZA^R-related genes direct their linkage with cryptic resistance mechanisms of PZA in *M. tuberculosis*.

Conclusion

In conclusion, to the best of our knowledge, this is the first study, covering the combined phenotypic and genotypic characterization of all PZA^R-associated genes in a wide range of *M. tuberculosis* clinical isolates. PZA^R rate among MDR (69%) and pre-XDR TB (61%) was considerably higher in southern China. Though *pncA* played a major role (>80%) in PZA^R but the simultaneous evaluation of other PZA^R related genes increased the sensitivity to ~90%. Considering the difficulty of phenotypic susceptibility testing method, our data suggest that concomitant detection of mutations in *pncA*$^+$ UFR, *rpsA, panD, Rv2783c,* and *Rv2044c* is more dependable. The addition of 57 novel mutations/indels from our study in drug-resistance TB mutation database may increase the reliability of molecular diagnosis. In addition, the substitutions at positions −11 and −12 in *pncA* UFR caused negative PZase activity but deletion of C nucleotide at −114 altered the effect of A−11G mutation and established the normal regulation of PZase, though the strain was still resistant to PZA. In addition, the loss of PZase activity due to newly identified mutations in Rv2044 led to confer PZA^R in *M. tuberculosis*. Lastly, the nine PZase positive PZA^R mutants bearing *pncA* synonymous mutations/indels and 26 PZA^R strains lacking any mutation in all reported PZA^R-related genes with discordant PZase activities implicate the existence of unknown resistance mechanisms that need to be uncovered.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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