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The epidemiology and gene mutation characteristics of pyrazinamide-resistant *Mycobacterium tuberculosis* clinical isolates in Southern China

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ABSTRACT

This study investigates the epidemic trend of pyrazinamide (PZA)-resistant tuberculosis in Southern China over 11 years (2012–2022) and evaluates the mutation characteristics of PZA resistance-related genes (*pncA, rpsA,* and *panD*) in clinical *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates. To fulfil these goals, we analyzed the phenotypic PZA resistance characteristics of 14,927 clinical isolates for which Bactec MGIT 960 PZA drug susceptibility testing (DST) results were available, revealing that 2,054 (13.76%) isolates were resistant to PZA. After evaluating the annual variation in the PZA resistance rate among tuberculosis cases in this region, it was observed that it decreased from 37.21% to 6.45% throughout the initial 7 years (2012–2018) and then increased from 8.01% to 12.12% over the subsequent 4 years (2019–2022). Sequences of *pncA* were obtained from 402 clinical *M. tuberculosis* complex isolates. For *rpsA* and *panD,* sequences were obtained from 360 clinical *M. tuberculosis* complex isolates. Mutations in *pncA* were found in 8 out of 223 PZA-sensitive isolates (3.59%) and 105 of 179 (58.66%) PZA-resistant isolates. Conversely, non-synonymous mutations in *rpsA* were identified in 5 of 137 (3.65%) PZA-resistant isolates, whereas the mutation ratio of *rpsA* among PZA-sensitive isolates was high at 14.03% (31/221). This difference in the *rpsA* mutation rate was statistically significant ($P = 0.001$, chi-square test). No *panD* mutations were observed in the 137 PZA-resistant isolates, whereas two PZA-sensitive isolates harboured point mutations in *panD*, including one nonsense mutation (C433 T) and another C-69 T mutation. These findings indicate that *rpsA* and *panD* may not significantly contribute to the development of PZA resistance in clinical *M. tuberculosis* isolates.

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Introduction

Tuberculosis (TB) is caused by the *M. tuberculosis* complex and remains one of the deadliest infectious diseases worldwide. In 2023, 10.8 million TB cases were diagnosed, and the TB incidence rate was 134 new cases per 100 000 population [1]. Therefore, efforts are needed to meet the objectives outlined in the "End TB Strategy," which aims for a 10% annual reduction by 2025, and an average annual reduction of 17% from 2025 to 2035.

Drug-resistant TB, particularly multidrug-resistant (MDR) TB, represents a major obstacle to achieving the goal of eradicating TB. Rational drug application and novel therapeutic design are vital in this context. Pyrazinamide (PZA) is valuable in the management of both drug-sensitive and MDR-TB, and its incorporation into the treatment regimens for drug-sensitive TB can reduce treatment duration from 9 to 6 months [2–5]. PZA can eliminate tubercule bacilli during the intensive chemotherapy phase, thereby improving clinical outcomes in MDR- and non-MDR-TB cases. Although technical and cost factors need to be considered, the World Health Organization recommends the BACTEC MGIT 960 system as the gold standard for *in vitro*

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analysis of PZA sensitivity [6–7]. Because not all patients with TB undergo PZA drug susceptibility testing (DST), the global epidemiology of PZA-resistant TB remains poorly understood despite evidence indicating that more than 50% of patients with MDR-TB are resistant to PZA [8]. Moreover, after adding PZA to the treatment regimen, the treatment failure rates were twice as high in patients with PZA-resistant MDR-TB compared to those with PZA-sensitive MDR-TB [9]. Efforts to reduce TB transmission and improve treatment efficacy have emphasized the need to develop reliable and rapid PZA DST to guide the clinical use of PZA.

Therefore, exploring the mechanisms underlying PZA resistance is vital for designing rapid molecular drug sensitivity tests. As a prodrug, PZA undergoes enzymatic conversion by pyrazinamidase (encoded by *pncA*) to produce its active pyrazinoic acid form. Mutations in *pncA* are believed to be the main cause of PZA resistance [5,10], and such mutations have been documented in almost all nucleotides of this gene [6, 11–13]. These *pncA* mutations primarily result in therapeutic resistance by reducing pncA protein levels, enzymatic activity, or both [14–15]. Although these *pncA* loss-of-function mutations are the leading causes of PZA resistance in *M. tuberculosis* isolates, up to 30% of PZA-resistant strains do not show any correlation between PZA resistance and mutations in *pncA* [16–18]. The significance of *rpsA* (encoding ribosomal proteins S1) and *panD* (encoding L-aspartate α-decarboxylase) mutation in TB resistance to PZA remains a matter of controversy [19–20], with some reports failing to detect any correlative relationship between PZA resistance and *rpsA* or *panD* mutations [21–25].

To date, most regions in China lack proper documentation of the true epidemiological features of PZA-resistant TB because of the restrictions on the use of PZA DST initiatives. Notably, including PZA in the treatment of patients with MDR-TB, particularly those who were PZA-sensitive, was associated with higher sputum culture conversion rates than that in patients who were PZA-resistant. Moreover, treating patients with PZA-resistant MDR-TB using PZA is always associated with higher treatment failure rates than those with PZA-sensitive MDR-TB [9, 26–28]. Consequently, effectively preventing and controlling MDR-TB remains a significant challenge. Developing a comprehensive understanding of the prevalence of PZA-resistant TB, particularly PZA-resistant MDR-TB, in this region is crucial for effectively managing and preventing TB. This knowledge is vital for clinical diagnosis and treatment.

To better explore the nature of the PZA-resistant TB epidemic in Southern China, this study retrospectively analyzed the epidemiological status of PZA-resistant TB in this region from 2012 to 2022. Moreover, sequencing analyses were conducted to identify mutations in *pncA*, *rpsA*, and *panD* and to examine their relationships with PZA resistance of clinical *M. tuberculosis* isolates in Southern China. Furthermore, an analysis was conducted on the mutation characteristics of genes linked to RIF and isoniazid (INH) resistance, such as *rpoB* and *katG*, to ascertain whether RIF and INH have any impact on PZA resistance of clinical *M. tuberculosis* complex isolates.

Materials and methods

Ethics approval

The study protocol was approved by the Ethical Review committee of Guangzhou Chest Hospital, and the need for informed consent was waived as this was a retrospective study.

Retrospective data and strain collection

This study retrospectively retrieved epidemiological data on 26,217 clinical isolates of the *M. tuberculosis* complex obtained from patients diagnosed with and treated for TB at Guangzhou Chest Hospital between January 2012 and October 2022. We selected this timeframe because it coincided with the period during which PZA DST was conducted at this institution and when this study was actively collecting data. *M. tuberculosis* complex was confirmed by MPT64 antigen testing, and resistance patterns for first-line anti-TB drugs were determined by the mycobacterial growth indicator tube (Bactec MGIT) 960 approach, a component of the Smart Cycler II System (Cepheid, USA), when the patients were diagnosed with TB. Since PZA DST was not routinely performed as part of first-line anti-TB DST, 11290 of the 26,217 clinical isolates were excluded due to the absence of PZA DST results at the time of TB diagnosis and treatment. Finally, 14,927 strains with available PZA DST results were retrospectively analyzed.

Subsequently, 402 clinical *M. tuberculosis* complex isolates, representing 11 different profiles of first-line anti-TB drug resistance, as shown in Figure 1 (Graphical Abstract) and Table 1, were selected for *pncA, rpsA*, and *panD* sequencing. Briefly, 7–99 strains were randomly selected from the Mycobacterial Biological Sample Bank.

The clinical and demographic characteristics of the patients, from whom the 14,927 strains (including the 402 sequenced isolates) were obtained, including sex, age, previous treatment, and anti-TB treatment schemes, were analyzed. The chi-square test was used to evaluate the relationship between PZA-resistant and prior treatment by comparing the differences in retreatment ratios between PZA-resistant patients and sub-groups of PZA-sensitive patients with the same resistance background to other first-line anti-TB drugs, including pan-sensitive, MDR, RIF mono-resistance, isoniazid (INH) mono-resistance, and streptomycin (STR) mono-resistance.

Further details of the study process are presented in Figure 1.

M. tuberculosis complex culture

This investigation included 402 clinical isolates of *M. tuberculosis* complex with different resistance patterns to first-line anti-TB drugs. Among these, 179 were PZA-resistant isolates, PZA-resistant isolates including 55 with PZA mono-resistance, 99 with

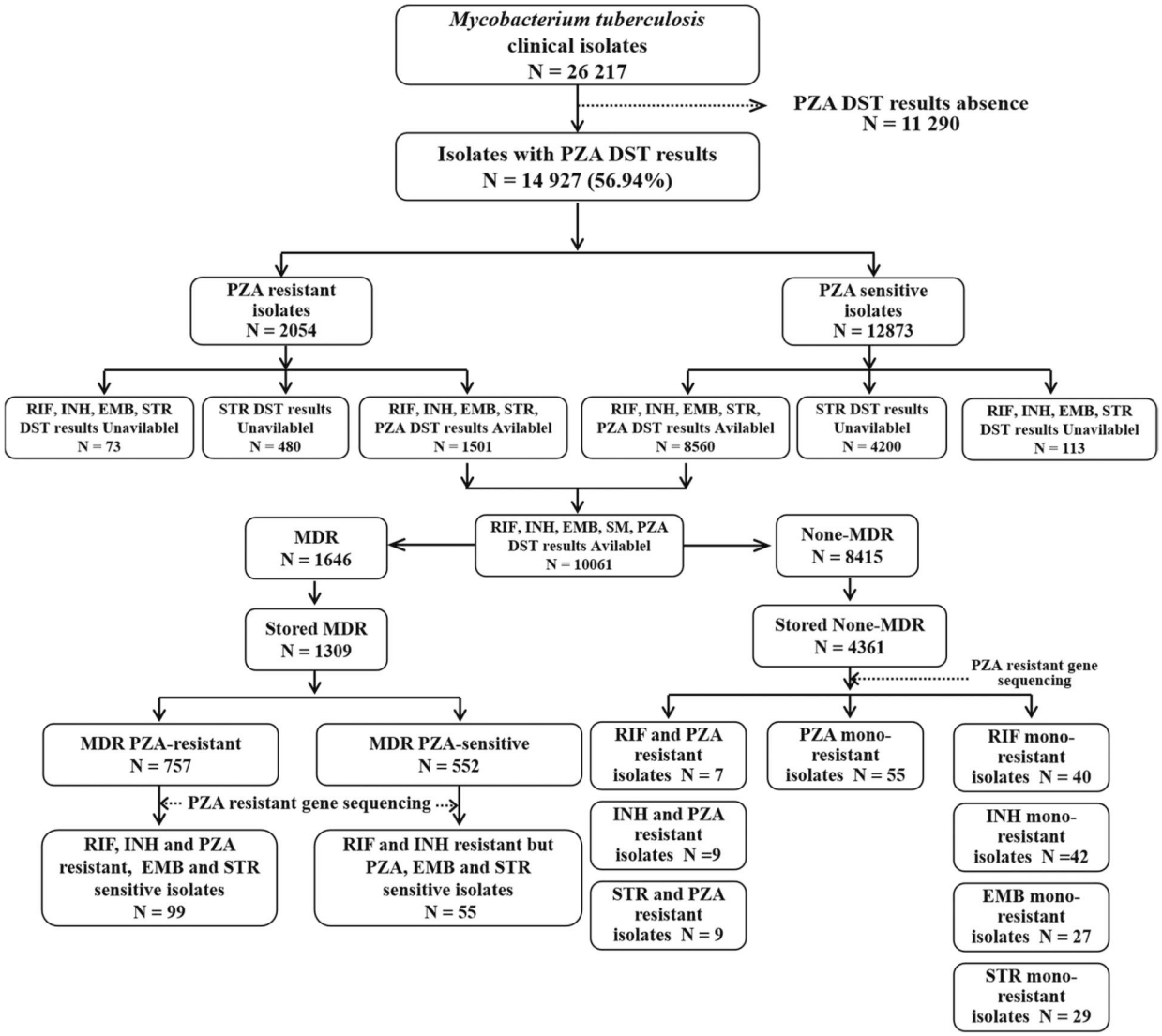

Figure 1. Flow chart of the study process (Graphical Abstract 1).

MDR, 9 with INH mono-resistance, 7 with RIF mono-resistance, and 9 with STR mono-resistance. The remaining 223 isolates were PZA-sensitive, comprising 30 pan-sensitive, 55 MDR, 42 INH mono-resistant, 40 RIF mono-resistant, 29 STR mono-resistant, and 27 ethambutol (EMB) mono-resistant strains. The target strains were stored at −80°C in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase complex (OADC) (Becton Dickinson, MD, USA) and 10% glycerol. Prior to in vitro analysis, the isolates were subcultured on 7H10 medium for 4 weeks at 37°C. All experiments were performed under enhanced biosafety level-2 (BSL-2) conditions, using appropriate laboratory equipment in accordance with national guidelines.

PZA, RIF, and INH resistance gene sequencing

The genomic DNA of freshly cultured *M. tuberculosis* complex isolates was extracted and stored at −20°C. The following primers were used for the amplification of *pncA, rpsA, panD, rpoB and katG*: *pncA*-F (5'-GTCGGTCATGTTCGCGATCG-3') and *pncA*-R (5'-GCTTTGCGGCGAGCGCTCCA-3'); *rpsA*-F (5'-CCGAGTTTGTCCAGCGTGTA-3') and *rpsA*-R (5'-CGTCATCTCGAAACGCCTTG-3'); *panD*-F (5'-TCAACGGTTCCGGTCGGCTGCT-3') and *panD*-R (5'-TATCCGCCACTGCTGCACGACCTT-3'); *rpoB*-F (5'-CGTACGGTCGGCGAGCTGATC-3') and *rpoB*-R (5'-AGGGGTTTCGATCGGGCACATC-3'); and *katG*-F (5'-TATACCGGACTACGCCGAAC-3') and *katG*-R (5'-ACCTGTCGAGGTTCATCACC-3'). Polymerase chain reaction (PCR) was performed in a total volume of 50 μL, containing 0.2 μL of Taq (5 U/μL), 5 μL of 10 × concentration of Taq Master Mix (Takara), 4 μL of dNTPs, 1 μL of F + R primers (10 μM), 1 μL (or 10 ng) of template DNA, and 37.8 μL of distilled H_2O. Cycling conditions were as follows: initial denaturation step at 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 62.5°C (*pncA*)/58°C (*rpsA*)/64°C (*panD*)/60°C (*rpoB*)/57°C (*katG*) for 45 s, 72°C for 1 min. The final extension was performed at 72°C for 5 min. Subsequently, the PCR products underwent sequencing, and the resulting data were evaluated using DNAMAN software for

Table 1. Mutation characteristics of pncA in clinical isolates of M. tuberculosis with different first-line anti-TB drug resistance profiles.

Drug resistance profile[a]	No.of isolates	Genotype	Amino acid changes (PZase)	Potential prevalence of PZA resistance[b]
P	45	WT[c]		
P	1	385–393 deletion	Frameshift	
P	1	457–465 deletion	Frameshift	
P	1	T→C at 104	L35P	Resistant
P	1	C→A at 129	H43Q	Resistant
P	1	C→T at 151	H51W	Resistant
P	1	C→G at 169	H57D	Resistant
P	1	A→G at 245	H82R	Resistant
P	1	T→G at 347	L116R	Resistant
P	2	C→T at 425	T142M	Resistant
HRP	13	WT		
HRP	1	20 T deletion	Frameshift	
HRP	1	52 C Insertion	Frameshift	
HRP	1	55–61 CTGGCGG Insertion	Frameshift	
HRP	1	62–108 deletion	Frameshift	
HRP	1	T→A at 62, 63–108 Deletion	Frameshift	
HRP	1[d]	182–191 deletion	Frameshift	
HRP	2	279 C Insertion	Frameshift	
HRP	1	280 G Insertion	Frameshift	
HRP	1[d]	281–288 deletion	Frameshift	
HRP	1	314 C Insertion	Frameshift	
HRP	1	315 G Insertion	Frameshift	
HRP	2[e]	316–330 TTCGAAGGAGTCGAC Deletion	Frameshift	
HRP	1	365–366 deletion	Frameshift	
HRP	1	385–393 deletion	Frameshift	
HRP	1	385 G deletion	Frameshift	
HRP	2	394 C Insertion		
HRP	2	391–395 deletion	Frameshift	
HRP	1	T→C at −12		
HRP	2	A→G at −11		
HRP	1	T→C at −7		
HRP	3	T→C at 14	I5T	Resistant
HRP	5	T→G at 20	V7G	Resistant
HRP	3	A→C at 29	Q10P	Resistant
HRP	2	G→A at 34	D12N	Resistant
HRP	1	T→C at 56	L19P	Resistant
HRP	2	C→A at 83	A28D	Resistant
HRP	2	A→G at 152	H51R	Resistant
HRP	3	C→T at 160	P54S	Resistant
HRP	1	C→A at 161	P54Q	Resistant
HRP	2	A→C at 226	T76P	Resistant
HRP	1	T→G at 269	I90S	Resistant
HRP	1	A→G at 286	K96E	Resistant
HRP	1	A→C at 287	K96T	Resistant
HRP	2	G→A at 290	G97D	Resistant
HRP	2	T→G at 307	Y103D	Susceptible
HRP	1	C→A at 312	S104R	Resistant
HRP	2	G→T at 313	G105C	Resistant
HRP	3[f]	A→C at 340	T114P	Resistant
HRP	1	T→G at 355	W119G	Resistant
HRP	1	C→T at 364	Q122→Stop	
HRP	5[g]	G→A at 395	G132D	Resistant
HRP	1[d]	A→G at 407	D136G	Resistant
HRP	1	G→A at 415	V139M	Resistant
HRP	1	T→C at 416	V139A	Resistant
HRP	2	T→G at 416	V139G	Resistant
HRP	1	C→T at 421	Q141→Stop	
HRP	3	A→C at 422	Q141P	Resistant
HRP	2	G→C at 427	A143P	Resistant
HRP	3	C→A at 437	A146V	Resistant
HRP	1	A→C at 457	T153P	Resistant
HRP	1	A→G at 212, A→C at 457	H71R, T153P	Resistant
HRP	1[d]	G→A at 485	G162D	Resistant
HP	6	WT		
HP	1	T→C at 2, G→A at 162	M1T + P54P	Susceptible[h]
HP	1	G→A at 41	C14Y	Resistant
HP	1	33 A insertion	Frameshift	
RP	3	WT		
RP	2	389–397 AGGTCGATG insertion	Frameshift	
RP	1	409 T insertion	Frameshift	
RP	1	C→G at 479	T160R	Resistant
SP	7	WT		
SP	1	C→T at 185	P62L	Resistant

(*Continued*)

Table 1. Continued.

Drug resistance profile[a]	No.of isolates	Genotype	Amino acid changes (PZase)	Potential prevalence of PZA resistance[b]
SP	1	T→G at 416	V139G	Resistant
HR	49	WT		
HR	1[d]	A→T at −11		
HR	2[e]	A→C at 29	Q10P	Resistant
HR	1[d]	C→T at 442	R148C	Susceptible
HR	1	392–393 GG insertion	Frameshift	
HR	1	A→C at 422	Q141P	Resistant
Q	28	WT		
Q	1	T→A at 52	S18T	Resistant
Q	1	C→T at 74	A25V	Susceptible
H	42	WT		
R	40	WT		
S	29	WT		
E	27	WT		

Note: [a] P, pyrazinamide, refers to PZA mono-resistance; H, isoniazid, refers to INH mono-resistance; R, rifampicin, refers to RIF mono-resistance; S, streptomycin, refers to STR mono-resistant; E, ethambutol, refers to EMB mono-resistance; HRP refers to INH, RIF and PZA resistance but EMB and STR sensitivity; HP refers to INH and PZA resistance but RIF, EMB and STR sensitivity; RP refers to RIF and PZA resistance but INH, EMB and STR sensitivity; SP refers to STR and PZA resistance but INH, RIF and EMB sensitivity; HR refers to INH and RIF resistance but PZA, EMB and STR sensitivity; Q, pan-sensitive to INH, RIF, EMB, STR and PZA; [b] potential prevalence of PZA resistance was suspected by SUSPECT-PZA web tool (https://biosig.lab.uq.edu.au/suspect_pza/submit_prediction); [c] WT, wild type; [d] the reference strains were XDR strains; [e] One of the two isolates was an XDR strain; [f] One of the three isolates was an XDR strain; [g] One of the five isolates was an XDR strain; [h] the prediction was based on the single nucleotide mutation (T2C, M1 T).

comparison with published sequences (GenBank accession number NC_000962).

Whole Genome Sequencing (WGS) was conducted for 55 of the 99 MDR PZA-resistant *M. tuberculosis* complex isolates to analyze the mutation characteristics of *pncA, rpsA, panD, rpoB* and *katG*.

Identification of Beijing genotypes M. tuberculosis

The deletion-targeted multiplex PCR (DTM-PCR) method [29] was applied to detect Beijing genotypes of *M. tuberculosis* strains. The following primers were used for amplifying the target gene with the genomic deletion RD105, which phylogenetically defines the Beijing family as a separate lineage within *M. tuberculosis* [29–30]: P1:5'-GGAGTCGTTGAGGGTGTTCATCAGCTCAGTC-3', P2:5' -CGCCAAGGCCGCATAGTCACGGTCG-3', P3:5' -GGTTGCCCACTGGTCGATATGGTGGACTT-3'. PCR was conducted in a total volume of 25 μL, containing 0.1 μL of Taq (5 U/μL), 2.5 μL of 10 × concentration of Taq Master Mix (Takara), 2.0 μL of 25 μM dNTPs, 0.2 μM of each primer and 1 μL (or 50 ng) of template DNA and 17.1 μl of distilled H_2O. Cycling conditions were as follows: 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min. A final extension at 72°C for 7 min was performed, and PCR products were separated on 0.8% agarose gels.

WGS was performed on 55 of the 99 MDR PZA-resistant *M. tuberculosis* complex isolates, and Beijing family strains were identified by examining the absence of RD105.

Statistical analysis

Data were analyzed using the SPSS (v.26.0) statistical software. Analyses were performed to determine the frequencies, percentages, ranges, and confidence intervals (CI) where necessary. Groups were compared using Fisher's test, with statistical significance defined as $P < 0.05$.

Results

The prevalence of PZA-resistant TB

This study enrolled 26,217 clinical *M. tuberculosis* complex isolates, of which 56.94% (14,927) had available Bactec MGIT 960-based PZA DST results. The average frequencies of PZA resistance among all isolates and the MDR and extensively drug-resistant (XDR) isolates were 13.76%, 56.88%, and 81.86%, respectively (Table 2). Among the other first-line anti-TB drug-sensitive or pan-sensitive strains, 3.24% were resistant to PZA (Table 2). When assessing the annual differences in PZA resistance ratios between MDR and pan-sensitive isolates from 2012 to 2022, the ratio was significantly higher in the MDR group ($P < 0.001$). Additionally, the PZA resistance rates of all clinical *M. tuberculosis* complex isolates were evaluated annually to provide insights into the evolution of PZA-resistant TB in Southern China. The rate declined from 37.21% to 6.45% over the initial 7 years (2012–2018) and increased from 8.01% to 12.12% over the next 4 years (2019–2022). A similar trend was evident among MDR isolates, which declined from 60.61% to 38.12% (2012–2018) before rising from 46.67% to 60.23% over the subsequent 3 years. For more detailed information, please refer to Table 2 and Figure 2.

The clinical and epidemiological characteristics of the enrolled patients with TB

The genetic characterization of PZA-resistant clinical TB isolates from Southern China was explored based on 402 clinical *M. tuberculosis* complex isolates.

Table 2. PZA resistance rates among clinical M. tuberculosis isolates and the prevalence of PZA resistance.

Year	Isolates Number	PZA DST tested isolates Number (%)	PZA resistance Number (%)	XDR-PZA resistant isolates Number (%)	MDR-PZA resistant isolates Number (%)	PZA mono-resistant isolates Number (%)
2012	225	129 (57.33%)	48 (37.21%)	5 (5/5, 100%)	20 (20/33, 60.61%)	10 (10/57, 17.54%)
2013	1218	741 (60.84%)	215 (29.02%)	16 (16/17, 94.12%)	95 (95/135, 70.37%)	58 (58/451, 12.86%)
2014	2318	1289 (55.61%)	281 (21.80%)	21 (21/28, 75.00%)	138 (138/204, 67.65%)	46 (46/719, 6.40%)
2015	3137	1994 (63.56%)	370 (18.56%)	37 (37/42, 88.10%)	190 (190/309, 61.49%)	55 (55/1165, 4.72%)
2016	3216	2056 (63.93%)	297 (14.45%)	42 (42/49, 85.71%)	195 (195/334, 58.38%)	16 (16/1090, 1.47%)
2017	3444	1745 (50.67%)	202 (11.58%)	10 (10/15, 66.67%)	129 (129/241, 53.53%)	23 (23/1184, 1.94%)
2018	2745	1534 (55.88%)	99 (6.45%)	0	69 (69/181, 38.12%)	8 (8/1105, 0.72%)
2019	3111	1198 (38.51%)	96 (8.01%)	5 (5/10, 50.00%)	56 (56/120, 46.67%)	11 (11/908, 1.21%)
2020	2686	1583 (58.94%)	133 (8.40%)	14 (14/17, 82.35%)	97 (97/175, 55.43%)	13 (13/1257, 1.03%)
2021	2413	1536 (63.66%)	177 (11.52%)	7 (7/10, 70.00%)	106 (106/176, 60.23%)	40 (40/1188, 3.37%)
2022	1704	1122 (65.85%)	136 (12.12%)	10 (10/11, 90.91%)	67 (67/135, 49.63%)	44 (44/862, 5.10%)
Total	26217	14927 (56.94%)	2054 (13.76%)	167 (167/204. 81.86%)	1162 (1162/2043, 56.88%)	324 (324/9986, 3.24%)

Clinical information of patients from whom these isolates were originally obtained and retrospectively collected for analysis is seen in Supplementary Table 1.

The retreatment ratios for PZA-resistant and PZA-sensitive TB patients with identical resistance profiles to other anti-TB drugs are present in Supplementary Tables 1 and 2. Notably, patients with PZA mono-resistance had a significantly higher retreatment ratio (17.56%) compared to pan-sensitive patients (0.00%, $P = 0.031$). Similarly, patients who exhibited resistance to both PZA and STR had a higher retreatment ratio (33.33%) than those who were resistant to STR alone (0.00%, $P = 0.040$) (Figure 3). However, among patients resistant to RIF and INH, whether simultaneously or individually, no statistically significant differences were observed between the PZA-resistant and PZA-sensitive groups ($P > 0.05$) (Figure 3).

The medical records of patients with TB, from whom the 14,927 clinical isolates were obtained and reviewed. As shown in Supplementary Figure 1C and Supplementary Table 3, about 65.65% of these patients were male. Additionally, the proportion of patients who relapsed consistently decreased annually from 15.50% to 6.65% throughout the initial 6 years (2012–2017), then fluctuated around 8% over the next 5 years (2018–2022). The percentages of the corresponding first-line anti-TB drug-resistant isolates, including XDR, MDR, pan-sensitive, PZA mono-resistant, INH mono-resistant, and RIF mono-resistant strains, were evaluated among all isolates each year. The percentage of pan-sensitive clinical *M. tuberculosis* complex isolates increased from 36.34% (2012) to 78.58% (2020) and then fluctuated around 74% in the next 2 years (74.74% in 2021 and 72.91% in 2022). For the composition of the clinical *M. tuberculosis* complex isolates with different first-line anti-TB drug resistance profiles, please refer to Supplementary Figure 1A and Supplementary Table 4.

pncA gene mutations

Differences in the frequency of *pncA* mutations were observed among PZA-resistant strains with different first-line anti-TB drug resistance patterns. Strains with monoresistance to RIF, INH, EMB, or STR were classified as non-MDR, while PZA-resistant strains were categorized into PZA mono-resistant, PZA-resistant MDR, PZA-resistant XDR, and PZA-

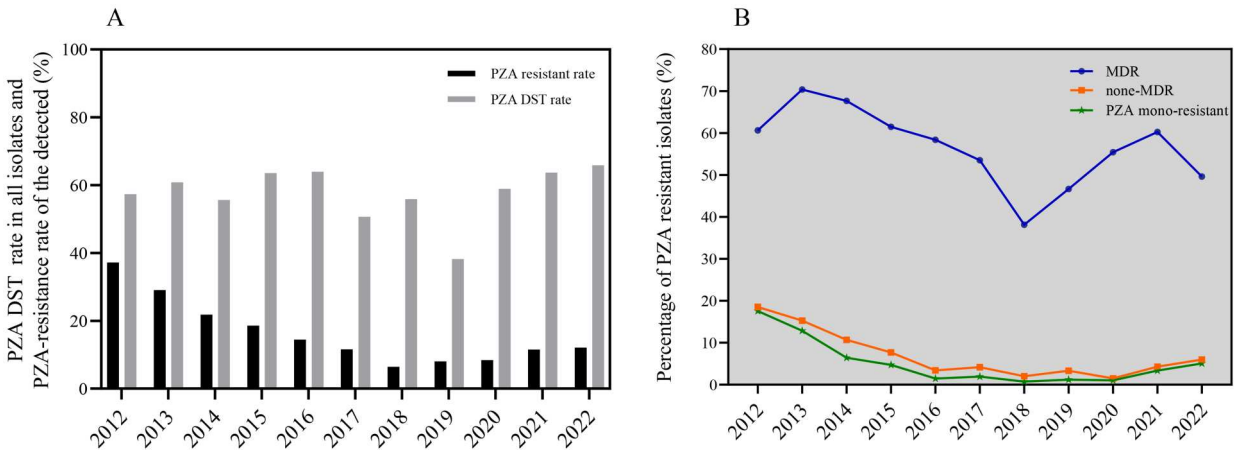

Figure 2. Epidemiological characteristics of PZA-resistant tuberculosis in southern China from 2012 to 2022. A: The PZA DST rate of all *M. tuberculosis* complex clinical isolates ($N = 26,217$) and the proportion reported as PZA-resistant among the isolates under DST detection. B: Proportions of PZA resistance among MDR, non-MDR and pan-sensitive isolates.

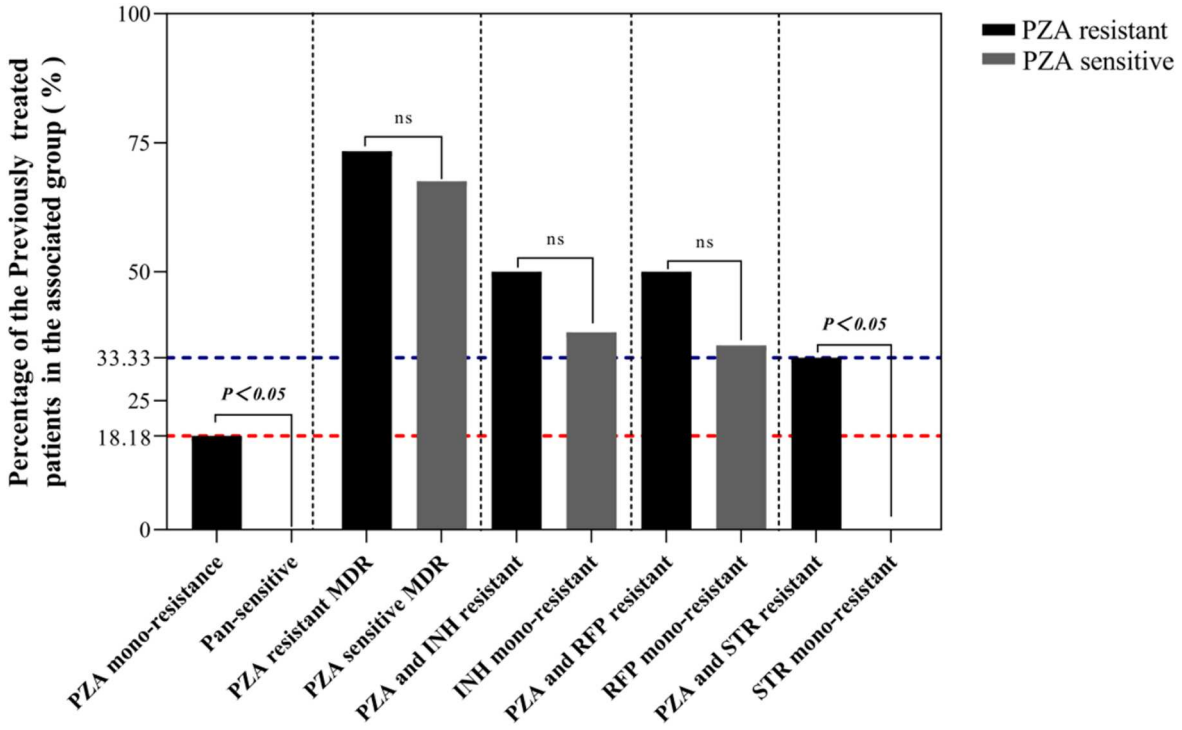

Figure 3. Percentages of previously treated patients in the associated drug resistance profile groups.

resistant non-MDR groups, with respective PZA resistance ratios of 18.18% (10/55), 85.87% (79/92), 100% (7/7), and 36.00% (9/25). The *pncA* mutation rate was significantly higher in the PZA-resistant MDR isolates than in the PZA-mono-resistant and non-MDR strains ($P < 0.001$, Figure 4). Additionally, the *pncA* mutation characteristics of the PZA-sensitive isolates were explored. First, none of the RIF-, INH-, EMB- or STR-mono-resistant isolates harboured *pncA* mutations. Subsequently, 6.67% (2/30) of the pan-sensitive isolates harboured *pncA* mutations. Ultimately, *pncA* mutations were found in 3 out of 52 PZA-sensitive MDR strains and all three PZA-sensitive XDR strains. Given the

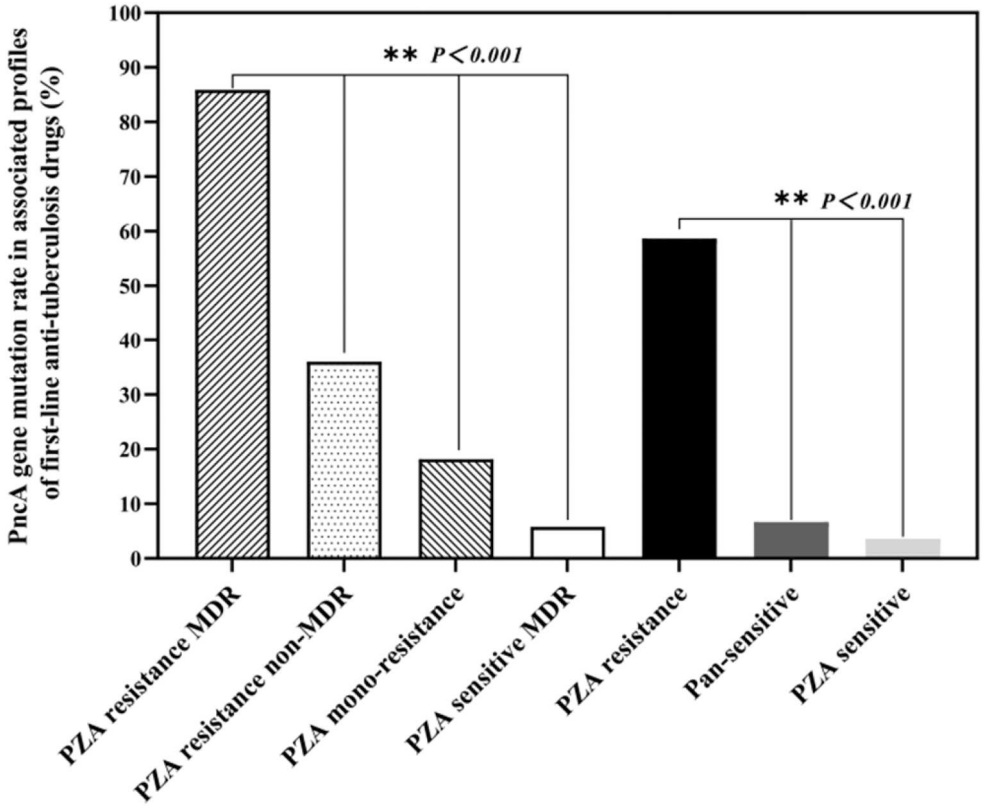

Figure 4. Rates of *pncA* mutations associated with resistance to the indicated first-line anti-TB drugs.

Table 3. Genotypic characteristics of *rpsA* mutation among the 360 clinical *M. tuberculosis* isolates.

Drug resistance profile	Isolates No.	Genotype	Amino acid changes
P	10	WT	
P	41	A→C at 636	R212R
P	1	548 G insertion	Framshift
P	1	T→C at 9	S3S
P	1	A→G at 135	K45K
P	1	G→A at 1318	A440T
HRP	10[a]	WT	
HRP	42[b]	A→C at 636	R212R
HRP	1	A→G at 135, A→C at 636	K45 K, R212R
HRP	1	C→T at 627	G209G
HRP	1	A→C at 636, G→A at 912	R212R, L304L
HRP	1	A→C at 636, C→A at 1319	R212R, A440E
HRP	1	1446 G insertion	Frameshift
HP	2	WT	
HP	5	A→C at 636	R212R
HP	1	A→C at 636, C→T at 1235	R212R, A412V
HP	1	C→T at 627	G209G
RP	4	WT	
RP	3	A→C at 636	R212R
SP	3	WT	
SP	6	A→C at 636	R212R
HR	8	WT	
HR	32	A→C at 636	R212R
HR	2	G→T at 239	V80G
HR	1	A→G at 135, A→C at 636	K45 K, R212R
HR	1	T→C at 137, A→C at 636, 618 T insertion	V46A + R212R + Frameshift
HR	1	G→C at 151, A→C at 636	V51L, R212R
HR	1	AA→TT at 358–359, A→T at 362, A→C at 636	K120L, E121 V, R212R
HR	1	A→C at 368, A→C at 636	D123A, R212R
HR	1	G→T at 56, C→T at 582, A→C at 636	S19I, T194 T, R212R
HR	1	567 C deletion, A→C at 636	Frameshift + R212R
HR	1	C→T at 587, A→C at 636	S196F, R212R
HR	1	GGC→CGG at 625–627, 652 G insertion	Frameshift + G209R
HR	1	5–7 deletion, A→C at 636	Frameshift + R212R
HR	1	589/597/627 C/T/A insertion, A→C at 636	Frameshift + R212R
HR	1	A→C at 636, C→T at 1323	R212R, G441G
HR	1	A→C at 636, GTG→CTT at 646–648, G→A at 831	R212R, V216L, K277K
Q	7	WT	
Q	1[c]	/	
Q	18	A→C at 636	R212R
Q	1	548 G Insertion, 1181 G insertion, A→C at 636, A→G at 1166	Frameshift + R212R + Q389R
Q	1	A→C at 636, C→A at 1319	R212R, A440E
Q	1	G→A at 1411	A471T
Q	1	1311–1313 deletion	Frameshift
H	9	WT	
H	26	A→C at 636	R212R
H	1	C→T at 193	A65T
H	1	T→C at 642	G214G
H	1	G→T at 56, A→C at 636	S19I, R212R
H	1	A→C at 368, A→C at 636	D123A, R212R
H	1	C→T at 582, A→C at 636	T194 T, R212R
H	1	A→C at 636, G→T at 689, G→T at 840, G→T at 885	R212R, G230 V, Q280H, Q295H
H	1	1171 A deletion, 1206 A deletion	Frameshift
R	8	WT	
R	29	A→C at 636	R212R
R	1	A→C at 636, T→G at 752	R212R, V251G
R	1	G→A at 1140	M380I
R	1	G→A at 1411	A471T
S	8	WT	
S	15	A→C at 636	R212R
S	1[c]	/	
S	1	C→T at 14	T5I
S	1	G→A at 193	A65T
S	1	A→C at 636, A→T at 806	R212R, P269L
S	1	A→C at 636, A→T at 989	R212R, H330L
S	1	G→T at 840, G→T at 885	Q280H, Q295H
E	2	WT	
E	22	A→C at 636	R212R
E	1	A→C at 636, G→T at 840	R212R, Q280H
E	1	G→T at 840, C→T at 853, G→T at 885	Q280H, R285W, Q295H
E	1	A→C at 636, A→C at 1389	R212R, S463S

Note: [a] 2 out of the 10 isolates were XDR strains; [b] 4 out of the 42 isolates were XDR strains; [c] *rpsA* gene could not be amplified in this isolate.

discovery of *pncA* mutations in PZA-sensitive isolates, along with the relatively low *pncA* mutation rate in PZA monoresistant strains, *pncA* mutation rates were compared between PZA monoresistant and pan-sensitive strains or between PZA monoresistant and all PZA-sensitive isolates. The findings

revealed a significantly higher *pncA* mutation rate in PZA-monoresistant isolates ($P<0.001$), as shown in Figure 4.

Of the 179 PZA-resistant strains, 101 (56.42%) harboured mutations located within *pncA*, including 75 (74.26%, 75/101) single nucleotide substitutions and 27 (26.73%, 27/101) frameshift mutations. Interestingly, 1 of the 75 PZA-resistant isolates simultaneously harboured single nucleotide substitution (T62A) and frameshift mutations (63–108 Deletion). Notably, 8 of the 223 (3.59%) PZA-sensitive strains harboured mutations located within *pncA*, including 7 (3.14%, 7/223) single-nucleotide substitutions and 1 (0.45%, 1/223) frameshift mutation. Of the seven PZA-sensitive isolates, one harboured a single-nucleotide substitution of A to T upstream of the *pncA* coding region (A-11 T). The remaining *pncA* mutations identified in the PZA-resistant and -sensitive isolates were distributed randomly across the 561 bp *pncA*, ranging from nucleotides 2–485 in these clinical isolates (Supplementary Figure 2). For more detailed information, refer to Tables 1 and 3.

rpsA gene mutation

PZA-sensitive isolates exhibited a significantly higher ratio of non-synonymous mutation of *rpsA* than PZA-resistant isolates (14.03% vs 3.65%, $P < 0.05$, Figure 5). Specifically, among the 137 PZA-resistant isolates for whom *rpsA* sequencing test was conducted, 5 harboured non-synonymous mutations in *rpsA* (Table 3), including 2 cases with insertions of G at position 548 or 1446, causing frameshift mutations, and 3 cases with distinct single nucleotide substitutions (C1235 T, G1318A, and C1319A). Of the 223 PZA-sensitive isolates, 2 were not amplified successfully.

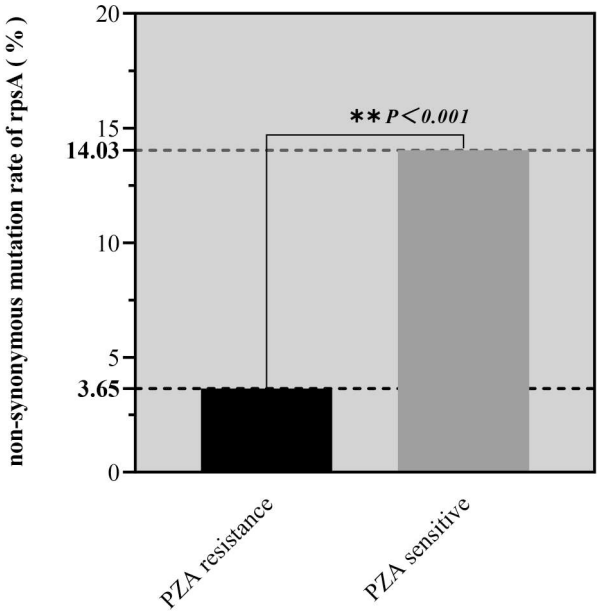

Figure 5. Comparisons of *rpsA* mutation ratios among PZA-resistant and PZA-sensitive isolates.

Among the remaining 221 strains, 31 harboured distinct non-synonymous mutations in *rpsA* (Table 3). Additionally, 266 of the 358 isolates harboured a common synonymous mutation (A636C), comprising 165 PZA-sensitive and 101 PZA-resistant isolates.

panD gene mutations

None of 137 PZA-resistant isolates, for whom *panD* sequencing was conducted, harboured *panD* mutations. However, mutations were detected in two PZA-sensitive isolates. These included one STR mono-resistant strain with a silent mutation (C433 T, Arg145Stop) and one MDR strain harbouring a mutation 69 bp upstream of *panD* consisting of a T substituted in place of a C. Both *panD* mutant strains exhibited wild-type *pncA* sequences and contained a synonymous mutation in *rpsA* (A636C). Moreover, the MDR strain exhibited a G151C mutation in the *rpsA*, resulting in a leucine substitution instead of valine.

rpoB and katG gene mutation

Of 99 MDR PZA-resistant isolates and 55 MDR PZA-sensitive isolates, 98 and 49 harboured *rpoB* mutations, respectively, and the ratio of S450L (C1349 T) mutation were significantly higher in MDR PZA-resistant isolates (77.78%, 77/99) than in MDR PZA-sensitive isolates (63.64%, 35/55), with *P* value of 0.046 ($P < 0.05$). For non-synonymous mutation details, please refers to Supplementary Table 5.

katG sequencing information was obtained for 64 of 99 MDR PZA-resistant isolates and 43 of 55 MDR PZA-sensitive isolates. S351 T and R463L were the most common non-synonymous mutations. The ratio of S315 T (G944C) mutation was significantly higher in MDR PZA-resistant isolates (85.94%, 55/64) than in MDR PZA-sensitive isolates (46.51%, 20/44, $P < 0.001$). No difference in R463L (G1388 T) mutation rate was observed between MDR PZA-resistant isolates (79.69%, 51/64) and MDR PZA-sensitive isolates (81.82%, 36/44). For a detailed information on *katG* mutation characteristics, please refer to Supplementary Table5.

Lineage information of the clinical M. tuberculosis isolates

The PCR products of 356 clinical *M. tuberculosis* complex isolates were either 761 bp (identifying a Beijing family strain) or 1466 bp (identifying a non-Beijing family strain) (Supplementary Figure 3). Beijing genotype identification was carried out on an additional 30 MDR PZA-resistant strains using WGS, which revealed that 74.87% (289/386) of the tested isolates belonged to the Beijing family. Moreover, sixteen samples failed to be identified for any target fragment,

including twelve PZA-resistant isolates and four PZA-sensitive isolates. Notably, 44.68% (129/289) of Beijing family strains exhibited resistance to PZA. Conversely, 39.18% (38/97) of the non-Beijing strains were resistant to PZA (Supplementary Table 6). However, statistical analysis revealed that the differences in the rate of PZA resistance between the Beijing and non-Beijing family strains were not significant, with a *P* value of 0.407 ($P > 0.05$). Additionally, 56.59% (73/129) of PZA-resistant and Beijing family strains obtained *pncA* mutation, and 60.53% (23/38) of the PZA-resistant and non-Beijing family strains carried *pncA* mutation. The differences in *pncA* mutation ratio of PZA-resistant isolates between Beijing and non-Beijing families were not significant, with a *P* value of 0.405 ($P > 0.05$).

Discussion

Owing to the limited use of PZA DST in all patients with TB, systematically or specifically clarifying the epidemic status of PZA-resistant TB worldwide, including in China, remains challenging. This study is an initial assessment of the epidemiological features of PZA resistance in Southern China over the past 11 years (2012–2022), revealing an estimated 13.76% (2054/14,927) of *M. tuberculosis* complex cases as PZA-resistant. Walker [31] used WHO-endorsed methods and reported approximately 14.6% PZA resistance among 15,903 clinical TB isolates. However, the PZA resistance ratio among patients with TB in different countries may vary. For example, in one report, PZA resistance rates in Azerbaijan, Bangladesh, Belarus, Pakistan, and South Africa ranged from 3.0% to 42.1% [32]. In countries with a relatively low incidence of TB, like the USA, the PZA resistance rate was estimated to be 2.7% (1999–2009) [33]. Budzik et al reported that 1.8% of *M. tuberculosis* isolates from San Francisco, CA, were PZA-resistant (1991–2011) [34]. In Jeddah, Saudi Arabia, the PZA resistance rate was slightly higher at 5.2% (2006–2016) [35].

Moreover, the epidemic trend of PZA-resistant TB remains unclear in most regions worldwide. Our study evaluated PZA resistance patterns among 14,927 clinical TB isolates collected over the past 11 years and found that the PZA resistance rate declined from 37.21% to 6.45% (2012–2018) before rising from 8.01% to 12.12% in the following 4 years (2019–2022). The epidemic trend of PZA-resistant TB might vary geographically, as reported: PZA resistance rates have risen over time, increasing from 0.2% to 2.2% in Canada (1988–1998) [36], 2.0% to 3.3% in the USA (1999–2009) [33], 1.6% to 3.6% in New York (2001–2008) [37], and 9.6% to 15% in China (2000–2010) [38]. However, the rate of PZA resistance in 2012 in our study was unexpectedly high (37.12%). Subsequently, a thorough reevaluation of the detailed DST data for the other three first-line anti-TB drugs was performed. Among these isolates, 29.69% were classified as MDR, with 65.78% being resistant to PZA. The high frequency of PZA resistance may be associated with the high prevalence of MDR isolates among the collected strains. Moreover, the resistance rate to PZA increased to 12.12% (2022), possibly owing to an increase in the burden of MDR-TB. Since PZA resistance was observed in more than 50% of patients with MDR-TB [39–41], this study compared the rate of PZA resistance in MDR and non-MDR isolates. The results showed that the rates of PZA resistance were significantly higher in the MDR isolates ($P < 0.001$). Other researchers have similarly reported lower PZA resistance levels among patients, with rates of 14.8%, 21%, and 20% reported in Pakistan [42], Uganda [43], and Tanzania [44], respectively.

This study aimed to determine whether RIF- and INH-resistant strains influence PZA resistance in *M. tuberculosis* complex clinical isolates. The PZA resistance ratio of non-MDR TB isolates was compared to that of MDR strains isolated annually from 2012 to 2022. Interestingly, the PZA resistance rate among MDR strains was significantly higher than that among non-MDR strains ($P < 0.001$). Matteo [32] determined that high PZA resistance rates were evident among individuals with RIF-resistant TB in Azerbaijan, Bangladesh, Belarus (Minsk), Pakistan, South Africa (Gauteng), and South Africa (KwaZulu Natal), with respective rates of up to 59.9%, 36.7%, 81.3%, 39.5%, 39.1%, and 49.1%. Conversely, the corresponding rates among RIF-sensitive isolates were substantially lower at 2.2%, 2.5%, 4.2%, 0.5%, 1.3%, and 1.3%. The higher rates of PZA resistance among patients with MDR-TB prompted an investigation into whether *pncA* mutation rates were similarly elevated in these MDR-TB strains. Upon comparing *pncA* mutation rate of MDR PZA-resistant strains (85.87%) with that of PZA-mono-resistant (18.18%) and PZA-resistant non-MDR strains (36.00%), we found a significantly higher *pncA* mutation ratios in MDR PZA-resistant isolates. The proportion of *pncA* mutations among MDR PZA-resistant strains in our study exceeded those reported in Ningbo (50.9%) [39], Fujian (63.6%) [45], Jiangsu (72.1%) [18], Beijing (79.3%) [23], and Chongqing (81.9%) [46], but was lower than the rates observed in Henan (89.52%) [12], Anhui (93.6%) [47], and Hunan (95.9%) [48]. This variation could potentially be attributed to the geographical origins of the sampled strains. Notably, 23 of the *pncA* nucleotide substitution mutations identified in our study were also reported in these regions.

However, the specific roles of RIF and INH in TB resistance to PZA remain poorly understood. In this study, we simultaneously detected *rpoB* and *katG* mutation of MDR PZA resistant and PZA sensitive

isolates. Surprisingly, we found that *rpoB* S450L mutation ratio was significantly higher in MDR PZA resistant isolates than in MDR PZA sensitive isolates. Moreover, *katG* S315 T mutation ratio was also significantly higher in MDR PZA resistant isolates than in MDR PZA sensitive isolates. Further research is need to explore the impact of *rpoB* and *katG* mutation on PZA resistant in *M.tuberculosis* isolates.

The genotypic background of *M. tuberculosis* may affect its PZA resistance. Our research found that Beijing family strains were prevalent in this area with no statistically significant difference in the PZA resistance rate between Beijing and non-Beijing family strains of TB. Moreover, no statistically significant difference in *pncA* mutation ratio was observed between the Beijing and non-Beijing family PZA-resistant isolates.

Another aim of this study was to determine the role of *rpsA* and *panD* mutations in PZA resistance of clinical *M. tuberculosis* complex isolates. Shi et al. [18–19] indicated that *panD* and *rpsA* may be targets of PZA, contributing to PZA resistance in strains encoding a wild-type *pncA* sequence [49–51]. The main finding of this study revealed an elevated mutation frequency of *rpsA* in isolates sensitive to PZA (14.03%) compared to those resistant to PZA (3.65%). Furthermore, *panD* mutations were exclusively present in the PZA-sensitive isolates and absent in the PZA-resistant strains. In a study conducted in Southern China, Tan et al. [51] observed *rpsA* mutations in *pncA* wild-type PZA-resistant TB isolates. Consistent with this finding, one *pncA* wild-type PZA-resistant isolate identified in this study harboured the *rpsA* mutation. Moreover, four PZA-resistant isolates harboured both *pncA* and *rpsA* mutations. In addition, two of the *rpsA* nucleotide substitution mutations (A368C, C1235 T) identified in our study were also reported in Beijing[23] and Henan[12]. Didem [52] found that *pncA/rpsA* and *pncA/panD* mutations coexisted in 12 and 2 isolates, respectively. However, the relevance of *panD* in PZA resistance to *M. tuberculosis* remains controversial. An analysis of genetic traits in 1,849 TB isolates from a public database identified eight strains harbouring *panD* mutations. Among these strains, only one was resistant to PZA, whereas the other seven were sensitive [24]. In this study, *rpsA* and *panD* mutations did not play an important role in PZA resistance in the clinical isolates of *the M. tuberculosis* complex.

Limitations

This study has a few limitations. First, the strains in our study were sourced exclusively from Southern China, therefore, our findings may not accurately reflect the overall frequency of PZA-resistant TB across the entire country. Conducting regional multicenter studies in the future would be crucial to fully understand the epidemiology of PZA-resistant TB in China. Additionally, given the storage conditions of the *M. tuberculosis* complex clinical isolates, several of the 11 groups mentioned in Table 1 had less than 50 strains. These factors may have influenced the results, possibly leading to a discrepancy between our findings and the actual prevalence of *pncA* mutations in non-MDR PZA-resistant *M. tuberculosis*. Future research should focus on increasing the number of strains within the respective categories to allow for a more thorough examination of clinical isolates of the *M. tuberculosis* complex. Moreover, another limitation is the absence of detailed diagnostic and treatment information for specific patients with TB corresponding to certain clinical isolates. Consequently, we could only assess patient information with complete records. Moreover, as this was a retrospective study, we could not randomly select isolates; instead, we included all isolates that had available PZA DST results. Hence, although our analysis indicated that drug-resistant TB patients (59.09%) had a slightly higher likelihood (by 2.07%) of undergoing PZA DST than pan-sensitive TB patients (57.02%) there may be a slight discrepancy between the observed prevalence of PZA resistance in our study and the actual prevalence in this region. However, given the marginal difference in PZA DST rates between these two groups, we believe this potential bias to be relatively low.

Conclusion

MDR clinical isolates showed a higher phenotypic resistance ratio to PZA, with the highest frequency of *pncA* mutations observed in MDR strains that were resistant to PZA. These findings suggest that *pncA* effectively explains PZA resistance in MDR strains. After sequencing all the 360 clinical isolates, whether they were *pncA* wild-type or mutant, the *rpsA* mutation ratio was significantly higher in PZA-sensitive isolates than in PZA-resistant strains. Notably, no *panD* mutations were detected in isolates resistant to PZA, whereas two PZA-sensitive strains carried *panD* mutations. Therefore, *rpsA* and *panD* may not significantly affect *M. tuberculosis* complex clinical isolates in Southern China.

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Disclosure statement

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