

Mycobacterium tuberculosis pncA Polymorphisms That Do Not Confer Pyrazinamide Resistance at a Breakpoint Concentration of 100 Micrograms per Milliliter in MGIT

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Sequencing of the *Mycobacterium tuberculosis pncA* gene allows for pyrazinamide susceptibility testing. We summarize data on *pncA* polymorphisms that do not confer resistance at a susceptibility breakpoint of 100 µg/ml pyrazinamide in MGIT within a cohort of isolates from South Africa and the U.S. Centers for Disease Control and Prevention.

Culture-based drug susceptibility testing (DST) using Bactec MGIT 960 PZA medium (Becton Dickinson, Sparks, MD) at 100 µg/ml is the current gold standard test for pyrazinamide (PZA) resistance (1). False resistance results are known to occur with this assay (1, 2), which may be the result of alkalinization of the medium due to a high inoculum size or the presence of bovine serum albumin (3). The uses of alternative susceptibility breakpoint concentrations or different media are additional factors that may contribute to disparities in PZA susceptibility results (4–6). A further limitation of culture-based methods is the long turnaround time, which can exceed 20 days (7–9). Molecular methods offer an alternative strategy for the detection of PZA susceptibility. These methods detect polymorphisms in the 561-bp *pncA* gene, which encodes the pyrazinamidase (PZase) enzyme that is responsible for conversion of PZA (prodrug) to pyrazinoic acid (active form) (10). More than 325 polymorphisms (single nucleotide polymorphisms [SNPs], insertions, and deletions) throughout the length of the *pncA* gene and in the promoter region have been described, complicating molecular detection (11–14). A good correlation (sensitivity of >90%) between *pncA* polymorphisms in circulating isolates and phenotypic susceptibility results have been observed for PZA (15–18). Incomplete correlation of *pncA* molecular results with culture-based PZA testing has been ascribed to poor reproducibility of the phenotypic test or the presence of alternative genetic mechanisms for resistance, including polymorphisms in the *rpsA* gene (19, 20). Additionally, a few *pncA* mutations have been reported in the absence of phenotypic resistance (15), but the role of such mutations in PZA resistance has not been thoroughly investigated. This study aimed to collate data on *pncA* polymorphisms in clinical isolates that do not confer resistance at a susceptibility breakpoint of 100 µg/ml PZA. To capture the spectrum of *pncA* mutations not associated with phenotypic PZA resistance, we performed a comprehensive literature search. In the 77 papers reporting genotypic and phenotypic PZA susceptibility (see Table S1 in the supplemental material), 77 different *pncA* polymorphisms in 71 different codons were reported to have a PZA-susceptible phenotype using either Bactec MGIT 960 PZA (Becton Dickinson, Sparks, MD), Bactec 460 PZA (Becton Dickinson, Sparks, MD) or the Wayne assay (21). Forty-seven (61%) of these polymorphisms have also been reported in PZA-resistant

isolates. These inconsistent phenotypic results may be due to technical difficulties of phenotypic PZA assays or MICs that are close to the breakpoint. Another 26 (33.7%) mutations were found in only one or two isolates, suggesting that these new mutations need to be characterized further to determine their role in PZA resistance.

To further investigate the relationship between *pncA* mutation and PZA susceptibility, we analyzed clinical isolates from culture collections at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA ($n = 185$) and Stellenbosch University, Stellenbosch, South Africa (SA) ($n = 865$). For the CDC isolates, *pncA* was previously sequenced, and only isolates with mutant *pncA* were included in this study. For the SA isolates, the *pncA* gene was amplified and sequenced using the ABI3130XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Polymorphisms in *pncA* were identified in 231 (26.7%) of the SA clinical isolates relative to those in the PZA-susceptible H37Rv reference strain. All isolates harboring *pncA* mutations (CDC and SA) were subjected to DST against PZA (BD PZA kit) at a critical concentration of 100 µg/ml using Bactec 960 MGIT. This phenotypic testing identified 7 of 185 (3.8%) CDC isolates and 42 of 231 (18.2%) SA isolates to have a susceptible PZA phenotype despite the presence of mutant *pncA* alleles. These results were confirmed by repeat *pncA* sequencing and repeat PZA DST. From these 49 isolates, 10 different *pncA* polymorphisms (synonymous, $n = 2$;

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TABLE 1 Single nucleotide polymorphisms in *pncA* found to not be associated with PZA drug resistance within a cohort of South African and U.S. isolates

Codon (nucleotide position, bp)	Nucleotide change	Amino acid change ^a	Associated MIC ($\mu\text{g/ml}$)	Source of isolate	No. of isolates
35 (104)	CTG→CGG	Leu→Arg ^b	>25 to <75	Stellenbosch	13
37 (110)	GAA→GTA	Glu→Val	50	CDC	2
65 (195)	TCC→TCT	Ser→Ser ^b	<25	Stellenbosch and CDC	21
96 (288)	AAG→AAA	Lys→Lys	<25	Stellenbosch	2
110 (329)	GAC→GGC	Asp→Gly	50	CDC	1
114 (341)	ACG→ATG	Thr→Met ^b	<25	Stellenbosch	5
130 (389) ^c	GTG→GCG	Val→Ala ^b	<25 and >50 to <100	Stellenbosch	2
163 (488)	GTG→GCG	Val→Ala	>75 <100	CDC	1
170 (509)	GCC→GTC	Ala→Val	75	CDC	1
180 (538)	GTC→ATC	Val→Ile	75	CDC	1

^a Ala, Alanine; Arg, Arginine; Asp, Aspartate; Glu, Glutamate; Gly, Glycine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Ser, Serine; Thr, Threonine; Val, Valine.

^b This mutation was also identified in a literature search.

^c This polymorphism had one isolate with an associated MIC of <25 $\mu\text{g/ml}$ and another isolate with an associated MIC of >50 and <100 $\mu\text{g/ml}$.

nonsynonymous, $n = 8$) were identified (Table 1). Six polymorphisms were newly identified in this study, while 4 corresponded to previously described *pncA* polymorphisms. Of these four, only Thr114Met was previously observed exclusively in susceptible isolates. The polymorphisms were not restricted to a defined domain but were broadly distributed throughout the *pncA* gene, with a distribution similar to that of resistance-causing mutations (22).

To further explore PZA resistance, each of the 49 PZA-susceptible isolates identified in this study was subjected to PZA MIC determination using PZA concentrations of 25, 50, 75, and 100 $\mu\text{g/ml}$ in Bactec MGIT 960 PZA medium. Six of the polymorphisms (all nonsynonymous SNPs) showed MICs between 50 and <100 $\mu\text{g/ml}$, 3 polymorphisms (2 were synonymous) were associated with MICs of <25 $\mu\text{g/ml}$, and 1 polymorphism had MICs of <25 for one isolate and >50 for a second isolate (Table 1). It is important to note that most of the *pncA* polymorphisms associated with susceptible isolates (7/10) identified in this study had a PZA MIC between 50 and 100 $\mu\text{g/ml}$. Six of 10 *pncA* polymorphisms associated with susceptibility were present in more than one isolate. The reproducibility of the MIC determinations across different clinical isolates with the same *pncA* mutation supports the notion that these polymorphisms do not confer resistance above the breakpoint concentration. However, some of the SNPs identified in this study were reported to confer resistance in other studies (see Table S1 in the supplemental material, references 1, 2, 24, 36, 50, 76). These conflicting results may be due to the PZA MICs for these isolates being close to breakpoint or associated with technical difficulties of performing PZA DST on solid media (1, 2).

We acknowledge that the clinical relevance of these polymorphisms on treatment outcome remain to be determined. In a recent report, an MIC of >50 $\mu\text{g/ml}$ and <100 $\mu\text{g/ml}$ was associated with a poor 2 month sputum conversion (relative risk of 1.5 [95% confidence interval, 1.2 to 1.8]) compared with that of an MIC of ≤ 50 $\mu\text{g/ml}$ (23). Accordingly, the authors concluded that a PZA susceptibility breakpoint of ~ 50 $\mu\text{g/ml}$ should be used for clinical decision making. However, one cannot exclude other factors that may have contributed to the observed delayed treatment response (23).

Based on the current accepted PZA susceptibility breakpoint concentration of 100 $\mu\text{g/ml}$ (1), not all *pncA* mutations necessarily confer resistance. We propose that genetic PZA drug susceptibility testing results should be interpreted based on known phe-

notype and genotype relationships at a susceptibility breakpoint of 100 $\mu\text{g/ml}$ until further evidence is presented to support any revision of the susceptibility breakpoint. Further studies are required to improve our understanding of the relationship between treatment outcome and *pncA* mutations.

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