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Association between *pncA* Gene Mutations, Pyrazinamidase Activity, and Pyrazinamide Susceptibility Testing in *Mycobacterium tuberculosis*

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We determined MICs for, confirmed the presence of *pncA* mutations in, and performed pyrazinamidase testing on colonies (subclones) obtained from seven isolates that exhibited differential pyrazinamide (PZA) susceptibility. Six of the seven strains were found to exhibit characteristics resulting from the mixture of strains possessing different properties. In addition, our analysis revealed large *pncA*-spanning deletions (1,565 bp, 4,475 bp, and 6,258 bp) in three strains that showed high PZA resistance.

Pyrazinamide (PZA) is an important drug used in the treatment of tuberculosis (1). The conversion of PZA to pyrazinoic acid (POA) by pyrazinamidase (PZase) is necessary for its activity. Bacterial resistance to PZA has been shown to correlate with mutations in the *pncA* gene that encodes PZase (2, 3, 4).

The antimicrobial activity of PZA is considerably affected by the pH of its environment (5). The Bactec MGIT 960 system (Becton, Dickinson, Sparks, MD) was used to measure PZA drug susceptibility (6, 7) in bacteria grown in liquid medium that was adjusted to pH 5.9 (8). However, the MGIT system has generated inconsistent test outcomes when assessing PZase activity and the presence of *pncA* mutations (9, 10, 11).

In this study, we evaluated PZA drug susceptibility in 83 multidrug-resistant *Mycobacterium tuberculosis* strains (recently isolated in Japan) by using MGIT and PZase testing, and we confirmed the presence of *pncA* mutations in these strains. The strains were examined for their susceptibility to 100 μg/ml of PZA by using an MGIT PZA antimicrobial susceptibility testing assay, as previously described (8). For the strains that demonstrated resistance to 100 μg/ml PZA, the PZA MIC was determined using the EpiCenter system (Becton, Dickinson) by dissolving PZA powder to 200, 300, 400, 800, and 1,600 μg/ml in MGIT culture medium. PZase analysis was conducted using Wayne's method (12).

Using PCR, *pncA* was amplified from each strain and then directly sequenced according to the methods described by Sreevatsan et al. (13). The 673-bp region spanning *pncA* and an 82-bp upstream sequence amplified using *pncA* primers (see Table S1 in the supplemental material) were purified using MagExtractor (Toyobo, Osaka, Japan). The PCR products were then directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3137 sequencer (Applied Biosystems). The *pncA* sequences were compared to the H37Rv strain genome (ATCC 27294) by using Genetyx Win v5.2 (Genetyx Co., Japan). In cases where PCR failed to yield a *pncA* amplicon, the strains were probed for amplification products upstream and downstream of *pncA* to investigate the cause of the negative results. PCR products were directly sequenced using the primer sets that were used for amplification.

The strains that differed with respect to *pncA* mutation or PZase activity at an MIC of ≥200 μg/ml and that were resistant to PZA were subcultured, and 10 subclones were randomly selected for each strain. Each subclone was again subjected to MIC and PZase testing and *pncA* mutation confirmation. The subclones were compared using a 15-locus variable-number tandem repeat (VNTR) analysis, as described by Supply et al. (14).

Three of the 83 strains were excluded from the study because of contamination. Of the remaining 80 strains, 31 (38.8%) showed susceptibility in the MGIT PZA assay, carried no *pncA* mutations, and tested positive for PZase, whereas 49 strains (61.2%) showed a PZA MIC of ≥200 μg/ml. Of these 49 strains, 39 (79.6%) carried a *pncA* mutation and were PZase negative (Fig. 1; see also Table S2 in the supplemental material). Colonies from 7 of the 49 strains (14.3%) which differed with respect to *pncA* mutation or PZase activity and exhibited a PZA MIC of ≥200 μg/ml were subcultured. Each subclone was again subjected to MIC and PZase testing and to *pncA* mutation confirmation (Table 1). Strain 4 displayed characteristics from two different subclones: (i) had a PZA MIC of ≤100 μg/ml, carried no *pncA* mutation, and was PZase positive, and (ii) had a PZA MIC of 1,600 μg/ml, carried a *pncA* mutation, and was PZase negative. Strains 13, 17, 50, and 79 displayed the following subclone characteristics: (i) had a PZA MIC of ≤100 μg/ml, and (ii) had a PZA MIC of ≥200 μg/ml, carried no *pncA* mutation, and were PZase positive. Strain 25 exhibited characteristics of three subclones: (i) had a PZA MIC of ≤100 μg/ml, carried no *pncA* mutation, and was PZase positive, (ii) had a PZA MIC of 400 μg/ml, carried no *pncA* mutation, and was PZase positive, and (iii) had a PZA MIC of ≥1,600 μg/ml, carried no *pncA* mutation, and was PZase negative. Strain 6 had an MIC of ≤100 μg/ml, showed no *pncA* mutation, and was PZase positive. No strain exhibiting an MIC of ≥200 μg/ml was isolated. VNTR analysis was conducted to compare the subclones from the six strains that exhibited mixed-strain characteristics. We found that the copy numbers were identical in each strain for each locus except for strain 17, which had a difference on QUB11b.

These results indicated that six of the seven strains exhibited

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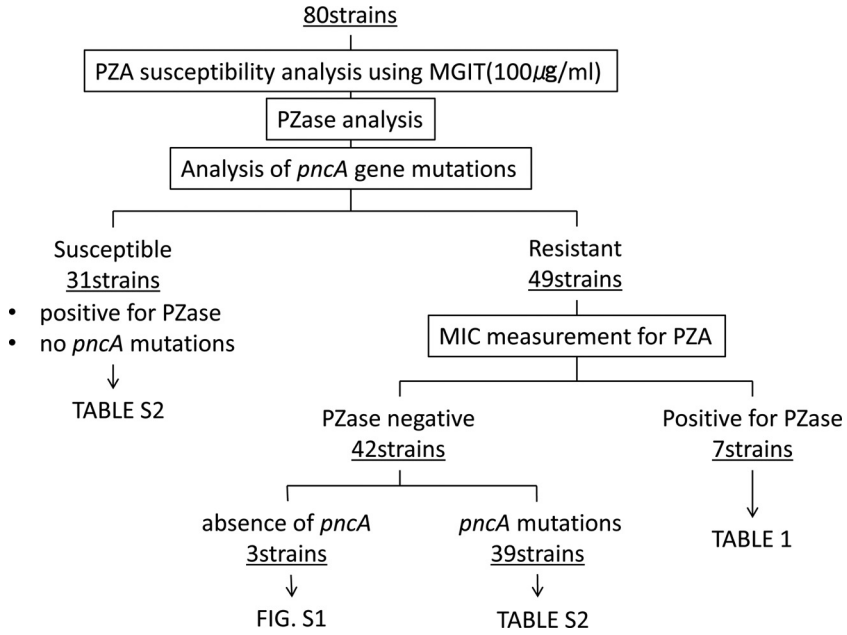

FIG 1 Flow diagram of PZA susceptibility analysis, *pncA* mutation testing, PZase analysis, and PZA MIC determinations (see also Fig. S1 and Table S2 in the supplemental material).

overall phenotypic characteristics resulting from the mixture of strains that possessed different properties. Strains 4 and 25 showed a mixture of positive and negative PZase test results, suggesting that these strains might have been deemed positive despite the presence of a PZase-negative strain (15). Strain 25 appeared to be a mixture of a *pncA* mutant and a wild-type strain, and the initial analysis was reflective of a wild-type strain. Shi et al. (16) identified the ribosomal protein S1, encoded by the *rpsA* mutation, as an alternative target of POA that might be associated with PZA resistance in the absence of mutations in *pncA*. Moreover, Sheen et al. (17) reported that the POA efflux rate is associated with PZA resistance. Thus, the six PZA-resistant strains without *pncA* mutations in this study might carry *rpsA* mutations or might have altered POA efflux.

Three strains failed to yield *pncA* PCR amplicons. Strains 33, 47, and 61 produced PCR products at Rv2040 and Rv2047, Rv2041 and Rv2045, and Rv2031 and Rv2046, respectively. Each of these strains carried a complete deletion of *pncA*, which, to the best of our knowledge, is a novel finding. In addition, all three strains had MICs of >1,600 μg/ml. Strains 33, 47, and 61 contained a 4,475-bp deletion from Rv2041 to Rv2046, a 1,565-bp deletion from Rv2042 to Rv2044, and a 6,258-bp deletion from

TABLE 1 Results of retesting isolated strains with divergent results from PZA MIC measurements using the Bactec MGIT 960 system, *pncA* gene sequencing, PZase analysis, and VNTR analysis

Original strain no.	Results for original strain			Isolated strain no.	Results for isolated strain			
	MGIT PZA MIC (μg/ml)	*pncA* mutation	PZase analysis		MGIT PZA MIC (μg/ml)	*pncA* mutation	PZase analysis	VNTR[a]
4	>1,600	G insertion at 392	Positive	i	≤100	None	Positive	Consistent in all loci
				ii	>1,600	G insertion at 392	Negative	
6	400	None	Positive	i	<100	None	Positive	
13	>1600	None	Positive	i	≤100	None	Positive	Consistent in all loci
				ii	>1,600	None	Positive	
17	400	None	Positive	i	≤100	None	Positive	QUB11b:7[b]
				ii	400	None	Positive	QUB11b:6[b]
				iii	800	None	Positive	QUB11b:7[b]
25	800	None	Positive	i	≤100	None	Positive	Consistent in all loci
				ii	400	None	Positive	
				iii	>1,600	T→G at 456, V139G	Negative	
50	200	None	Positive	i	≤100	None	Positive	Consistent in all loci
				ii	400	None	Positive	
79	200	None	Positive	i	≤100	None	Positive	Consistent in all loci
				ii	400	None	Positive	

[a] Compared to results for the isolated strain among the Supply et al. (14) 15-locus VNTR.
[b] Recognized a difference in the copy number at QUB11b.

Rv2037 to Rv2045, respectively (see Fig. S1 in the supplemental material). These results suggest that the entire *pncA* was deleted in *M. tuberculosis*, demonstrating that strains lacking *pncA* can still be PZA resistant.

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