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# Correlation between Pyrazinamide Activity and *pncA* Mutations in *Mycobacterium tuberculosis* Isolates in Taiwan

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**A total of 76 clinical *Mycobacterium tuberculosis* isolates from Taiwan were tested for pyrazinamidase activity, pyrazinamide susceptibility, and *pncA* mutations. Frequency of resistance to PZA rose with increases in resistance to first-line drugs. Of 17 pyrazinamide-resistant strains, 7 (3 of which had not been previously described) possessed mutations in the *pncA* gene.**

Pyrazinamide (PZA) has become increasingly important because of its ability to enhance the efficacy of isoniazid and rifampin and to allow shorter courses of therapy (2, 8, 20, 21). The unique feature of PZA is believed to be its ability to kill a population of semidormant tubercle bacilli that reside in acidic inflammatory environments (6). Unfortunately, susceptibility testing for PZA is still not sufficiently standardized to help guide therapy (7, 17, 19). Accordingly, there is a major need for more rapid and reliable tests. One approach is to detect mutations in the *pncA* gene. It has been reported that these mutations correlate well with a MIC of PZA of >100 μg/ml, with the frequency of *pncA* mutation among resistant strains (depending on the geographic area) ranging from 66.7 to 96.8% (1, 3–5, 8–12, 13–14, 18).

This study was designed to determine the frequency of PZA-resistant strains with *pncA* mutations among PZA-resistant and -susceptible *M. tuberculosis* strains isolated in Taiwan. In vitro susceptibility to PZA was correlated with PZase activity and the composition of the entire *pncA* nucleotide sequence. The frequency of PZA resistance among strains with various patterns of resistance to first-line drugs (isoniazid, rifampin, streptomycin, and ethambutol) was also investigated.

A total of 76 *M. tuberculosis* strains with variant drug susceptibility patterns isolated from 1994 to 2000 from clinical specimens collected in Kaohsiung Veterans General Hospital, Taiwan, were randomly selected. They included 27 strains susceptible to the four first-line antimycobacterial drugs, 28 multidrug-resistant (MDR) strains, and 21 strains with variable drug resistance patterns.

PZA MICs were tested by a BACTEC MGIT 960 PZA system because it requires no radioactive materials and has been shown to be as reliable as the BACTEC 460TB system (16). The susceptibility tests were performed at 100 and 300 μg/ml according to the manufacturer's instructions. The critical concentration of PZA for determination of resistance recommended by the manufacturer is 100 μg/ml. All the PZA-resistant strains were retested.

The PZase activity was assayed using the Wayne method (22). Several loopfuls of colonies were in two tubes of medium for each strain; one tube was examined after 7 days of incubation, and the other was examined after 14 days of incubation. All the strains that initially lacked PZase activity were retested.

DNAs were extracted with a Qiagen MinElute PCR purification kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions and stored at 4°C. A 720-bp region that included the entire open reading frame of *pncA* and 82 bp of an upstream putative regulatory sequence was amplified by PCR with the forward (P1) and reverse (P6) primers (GenBank accession number U59967; published by Scorpio) (18). A GeneAmp system 9600 thermocycler (Perkin-Elmer Corp., Foster City, Calif.) was used for target amplification with the following parameters: 5 min at 4°C followed by 30 cycles of 60 s at 94°C, 30 s at 63°C, and 60 s 72°C and termination with a final extension step at 72°C for 10 min. The PCR products were purified with the Qiagen MinElute PCR purification kit according to the manufacturer's instructions. The purified PCR products were sequenced in an ABI PRISM 310 genetic analyzer (Applied Biosystems, Inc., Foster City, Calif.). DNA sequencing reactions were performed with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc.). Sequence data were compared with a published sequence for *pncA* (GenBank accession number U59967). Differentiation of *M. tuberculosis* from other members of *M. tuberculosis* complex was done using specific deletion profiles (15).

A total of 59 of the 76 isolates were PZA susceptible; they all had identical wild-type *pncA* sequences. Among the 17 PZA-resistant isolates, 7 had a *pncA* nucleotide sequence change and lacked PZase activity (Table 1) (isolates 1 to 7). Three of these mutations (a deletion of nucleotides 352 to 358, a G insert at nucleotide 397, and a Phe 94→Ser mutation) have not been previously reported. The Phe 94→Ser mutation was located in one of the clusters of hydrophobic residues which are close to Lys 96 and which point towards the active-site region (11). Of the resistant strains, 10 carried wild-type *pncA*. One strain (isolate 8) had a wild-type *pncA* sequence without PZase activity. The strain

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TABLE 1. PZase activity and changes in the *pncA* gene for 76 clinical *M. tuberculosis* isolates

| Isolate | PZA MIC (μg/ml) | PZase activity after: | | Change in: | |
|---|---|---|---|---|---|
| | | 7 days | 14 days | Nucleotide(s) | Amino acid(s) |
| 1 | >300 | − | − | C→T at position 401 | F134→F |
| 2 | >300 | − | − | G→A at position 290 | G97→D |
| 3 | >300 | − | − | G→C at position 281 | F94→S |
| 4–5 | >300 | − | − | T→G at position 464 | V155→G |
| 6 | >300 | − | − | 352–358 deletion | Frameshift |
| 7 | >300 | − | − | 397 insert G | Frameshift |
| 8 | 300 | − | − | None | Not applicable |
| 9 | >300 | − | + | None | Not applicable |
| 10–11 | >300 | + | + | None | Not applicable |
| 12–17 | 300 | + | + | None | Not applicable |
| 18–76 | ≤100 | + | + | None | Not applicable |

was MDR and formed small colonies on a 7H11 agar plate. A negative reverse transcription-PCR result indicated that there could be a mutation of a *pncA*-regulatory gene and that this mutation could affect expression of *pncA*, thereby causing PZA resistance. One strain (isolate 9) had reduced PZase activity, giving negative PZase results at 7 days but giving positive results at 14 days; the other eight strains (isolates 10 to 17) had normal PZase activity. There might be mechanisms of PZA resistance that do not affect or diminish PZase activity or expression, such as mutations leading to modification or amplification of the pyrazinoic acid (POA) target or to enhanced POA efflux. These strains may provide an opportunity to further study the alternative mechanisms of PZA resistance.

Pyrazinamidase resistance were detected in 12 of 28 MDR strains, 3 of 21 strains resistant to all of the drugs tested, and 2 of 27 strains susceptible to the four first-line antimycobacterial drugs (Table 2). We found a rise in the frequency of resistance to PZA as the number of strains resistant to the first-line drugs increased. The emergence of resistance to the first-line antituberculosis drugs has led to an increased use of PZA and other drugs to combat resistance. This finding emphasizes the importance of PZA susceptibility testing.

We found a strong (98.7%) correlation between the loss of PZase activity and the presence of a *pncA* genotype. We were surprised to find that only 7 out of 17 PZA-resistant strains possessed a mutation in the *pncA* sequence and low (88.2%) correlation between the loss of PZase activity and PZA susceptibility. This finding is in contrast to prior reports (1, 3–5, 8–12, 13–14, 18). This method is therefore not sufficiently sensitive to be used as a surrogate marker for PZA resistance in Taiwan. Nevertheless, in view of the problems of standardization with the in vitro susceptibility tests we suggest that strains with borderline or poorly reproducible susceptibility should be examined for PZase activity and that rapid automated *pncA* DNA sequencing should be used.

TABLE 2. Frequency of PZA-susceptible strains according to frequency of susceptibility to first-line drugs

| Characteristics of resistance to first-line drugs other than PZA | No. of strains | No. (%) of strains with PZA: | | |
|---|---|---|---|---|
| | | Susceptibility (MIC ≤ 100) | Resistance | |
| | | | MIC = 300 | MIC > 300 |
| No resistance | 27 | 25 (92.6) | 1 (3.7) | 1 (3.7) |
| Resistance to all drugs tested | 21 | 18 (85.7) | 2 (9.5) | 1 (4.8) |
| MDR | 28 | 16 (57.1) | 4 (14.3) | 8 (25.6) |

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