

pncA Mutations as a Major Mechanism of Pyrazinamide Resistance in *Mycobacterium tuberculosis*: Spread of a Mono-resistant Strain in Quebec, Canada

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Pyrazinamide (PZA) is an important first-line tuberculosis drug that is part of the currently used short-course tuberculosis chemotherapy. PZA is a prodrug that has to be converted to the active form pyrazinoic acid by pyrazinamidase (PZase) activity, encoded by the *pncA* gene of *Mycobacterium tuberculosis*, and loss of PZase activity is associated with PZA resistance. To further define the genetic basis of PZA resistance and determine the frequency of PZA-resistant strains having *pncA* mutations, we sequenced the *pncA* gene from a panel of 59 PZA-resistant clinical isolates from Canada, the United States, and Korea. Two strains that did not contain *pncA* mutations and had positive PZase turned out to be falsely resistant. Three PZase-negative strains (MIC, >900 µg of PZA per ml) and one PZase-positive strain (strain 9739) (MIC, >300 µg of PZA per ml) did not have *pncA* mutations. The remaining 53 of the 57 PZA-resistant isolates had *pncA* mutations, confirming that *pncA* mutation is the major mechanism of PZA resistance. Various new and diverse mutations were found in the *pncA* gene. Interestingly, 20 PZA-mono-resistant strains and 1 multidrug-resistant isolate from Quebec, Canada, all had the same *pncA* mutation profile, consisting of an 8-nucleotide deletion and an amino acid substitution of Arg140→Ser. Strain typing indicated that these strains are highly related and share almost identical IS6110 patterns. These data strongly suggest the spread of a PZA-mono-resistant strain, which has not previously been described.

Pyrazinamide (PZA) is an important first-line tuberculosis (TB) drug. Along with isoniazid (INH), rifampin (RMP), and ethambutol (EMB), PZA is part of the currently used short-course treatment regimen, also called DOTS (for directly observed therapy, short course) recommended by the World Health Organization (21). PZA plays a unique role in achieving this shortened therapy, because PZA is believed to kill a population of semidormant tubercle bacilli residing in an acidic environment (e.g., as in active inflammation sites with low pH) in vivo that may not be affected by other TB drugs (12). Despite its role in shortening the TB therapy, PZA has no apparent activity against tubercle bacilli under normal pH conditions (18); the activity is only present at acidic pH (9). We have recently shown that the role of acid pH is to enhance accumulation of pyrazinoic acid (POA), the active moiety of PZA, in tubercle bacilli, whereas little POA accumulates in the bacterial cells at neutral pH (24). Structurally, PZA is an analog of nicotinamide. Like isoniazid (23), PZA is a prodrug. It requires conversion to POA by bacterial pyrazinamidase (PZase) in order to affect the tubercle bacilli (5, 14). Loss of PZase activity is observed in *Mycobacterium tuberculosis* strains that are resistant to PZA (5), and indeed, there is a very good correlation between PZA resistance and loss of this enzyme activity (8, 10, 11, 19). More details on PZA are given in a recent review by one of us (2).

To determine the genetic basis of PZA resistance, we have

identified the PZase gene (*pncA*) from *M. tuberculosis* (14) and have shown in a previous study that *pncA* mutations appear to be a major mechanism of PZA resistance (15). Forty-one of 42 PZA-resistant strains were found to have *pncA* mutations (15). Subsequent studies have confirmed these findings (4, 6, 7, 10, 15). However, a study by Sreevatsan et al. reported that only 72% of 67 PZA-resistant strains had *pncA* mutations (16). It is not clear whether the lower percentage of PZA-resistant strains with *pncA* mutations is due to incorrect PZA susceptibility testing such that a portion of “PZA-resistant” strains are actually susceptible (falsely resistant) or is a genuine finding. In fact, the currently used methods for PZA susceptibility testing are unreliable and problematic (3) because of insufficient standardization of the available tests (1). A rapid molecular test for PZA resistance based on detecting *pncA* mutations could circumvent the problems of conventional PZA susceptibility testing. An accurate picture of the percentage of PZA-resistant strains having *pncA* mutations is useful not only for understanding the mechanism of PZA resistance but also for developing a PCR-based test for rapid detection of PZA resistance by detecting *pncA* mutations. To achieve these goals, we have in the present study analyzed more PZA-resistant clinical isolates of *M. tuberculosis* in terms of the correlation between PZA resistance and *pncA* mutations. New and diverse *pncA* mutations were again found in PZA-resistant strains. An interesting finding is that many clinical isolates from Quebec, Canada, are PZA mono-resistant and they all share the same *pncA* mutation profile. IS6110 fingerprinting analysis indicated that these strains are highly related and suggest an active transmission of the disease by a PZA-mono-resistant *M. tuberculosis* strain.

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MATERIALS AND METHODS

Strains, PZA susceptibility testing, and PZase assay. Thirty PZA-resistant strains, 21 of which were mono-resistant, were from Quebec, Canada. These Canadian strains were collected over 3 years between 1990 and 1992 from 15 hospitals in 10 different regions of Quebec. The PZA-resistant *M. tuberculosis* strains were identified by using the pH 6.0 liquid medium in the BACTEC radiometric method with PZA concentrations of 100, 300, and 900 µg/ml for all of the U.S. strains (1), 100 and 300 µg of PZA per ml by the same method for the Canadian strains, or Lowenstein-Jensen medium at pH 5.6 with 100 and 500 µg of PZA per ml for the South Korean strains (strain designations starting with K). PZA resistance was defined as resistance to at least 100 µg of PZA per ml for the Lowenstein-Jensen method and the BACTEC method. For PZA susceptibility testing, susceptible strain H37Rv and PZA-resistant strain BCG were included as susceptible and resistant controls. PZase activity was assayed using the Wayne method (14) and confirmed using the C14-pyrazinamide method (17). A PZase-positive culture (PZA-susceptible *M. tuberculosis* strain H37Rv) and a PZase-negative culture (BCG Pasteur) were included as controls for the PZase assay.

Genomic DNA, PCR, and DNA sequencing. *M. tuberculosis* cultures were grown in 7H9 liquid medium with albumin-dextrose-catalase enrichment (Difco) at 37°C for 3 to 4 weeks. Genomic DNA isolation and PCR were performed as described previously (22). The *pncA* forward primer 5'GTCGGTCATGTTCCGCGATCG3' was from bp -105 upstream of *pncA*, which contains putative promoter region, and the reverse primer 5'GCTTTGCGGCGAGCGCTCCA3' was from 60 bp downstream of stop codon of the *M. tuberculosis pncA* gene (558 bp) (accession number U59967) (14). The expected size of the *pncA* PCR products was 720 bp. The *pncA* PCR products were run on Tris-borate-EDTA-0.8% agarose gel, and the DNA was isolated using a Qiagen kit according to manufacturer's instructions. The gel-purified PCR products were directly sequenced in an ABI automatic DNA sequencer (model 377), using the above-described forward and reverse primers.

IS6110 fingerprinting. Mycobacterial genomic DNAs from various PZA-resistant strains were digested with *PvuII* and then run on a Tris-borate-EDTA-0.8% agarose gel. The IS6110 probe used in the Southern hybridization was a 245-bp PCR DNA fragment amplified by PCR using INS-1 (5'CGTGAGGGC ATCGAGGTGGC3') and INS-2 (5'GCGTAGGCGTGGTGACAAA3') primers as described previously (20). The 245-bp PCR product was labeled with [³²P]dCTP using a random primer labeling kit (GIBCO BRL). The Southern blotting procedure was performed as described previously (22).

RESULTS

Identification of *pncA* mutations in PZA-resistant *M. tuberculosis* clinical isolates. To further define the molecular basis of PZA resistance and to determine the frequency of *pncA* mutations among PZA-resistant strains, we analyzed 59 PZA-resistant *M. tuberculosis* clinical isolates for potential mutations in the *pncA* gene by PCR sequencing (Table 1). Two strains (11830 and 10274) which were initially reported as resistant to PZA by using the BACTEC method at a PZA concentration of 100 µg/ml were in fact susceptible to PZA (falsely resistant) (MIC, <100 µg/ml) upon retesting. Fifty-three of 57 genuinely PZA-resistant strains had various *pncA* mutations, as shown in Table 1. The nature of the *pncA* mutations ranged from nucleotide transitions or transversions causing amino acid substitutions to nucleotide insertions or deletions causing nonsense polypeptides. It is remarkable that 17 new and diverse mutations were found in the 558-bp-long *pncA* gene (Table 1). Some of the strains had the same type of mutations but different IS6110 patterns, indicating that they are actually different strains which happened to acquire the same type of mutation. For example, strains T63168 and M52997 had the same mutation of Gly97→Ser, yet IS6110 analysis indicated that they are different TB strains (Fig. 1, lanes 1 and 2). Similarly, strains H2374 and H1033 both had a single nucleotide G insertion at position 420, but IS6110 typing showed that they are different strains (Fig. 1, lanes 3 and 4). Three PZA-resistant PZase-negative strains (11552, F57636, and M49586), for which the MICs were greater than 900 µg/ml, did not have any mutations in the *pncA* gene. Further sequence analysis of the *pncA* upstream region (2 to 3 kb before the start codon), which contains the putative *pncA* promoter, also failed to reveal any mutations (data not shown). This suggests that there could be

a *pncA* regulatory gene and that mutation of this gene could affect the expression of the *pncA* gene. One PZA-resistant strain (strain 9739) with positive PZase and for which the PZA MIC was 300 µg/ml did not have any *pncA* mutation.

Active transmission of a PZA-mono-resistant strain in Quebec, Canada. Twenty-one of 27 PZA-resistant clinical isolates from Quebec, Canada, were found to have the same type of *pncA* mutations, which consisted of an 8-bp deletion at nucleotide position 446 followed by an amino acid substitution of Arg140 to Ser. One such strain, 9953, is a multidrug-resistant (MDR) strain, which is resistant to INH, RMP, and EMB in addition to PZA. This suggests that this MDR strain initially had PZA mono-resistance but later acquired resistance to other drugs. IS6110 strain typing showed that the PZA-mono-resistant strains that had the characteristic 8-bp deletion and an amino acid substitution shared almost identical banding pattern (Fig. 1, lanes 5 to 8), indicating that these strains are highly related and were derived from a single source.

DISCUSSION

The present study has shown that 53 of 57 PZA-resistant *M. tuberculosis* clinical isolates had mutations in the *pncA* gene, indicating that *pncA* mutation is the major mechanism of PZA resistance in *M. tuberculosis*. The nature of the *pncA* mutations includes (i) substitution of amino acids due to nucleotide transitions or transversions or (ii) nucleotide insertions or deletions leading to nonsense polypeptides. The distribution of *pncA* mutations was dispersed along the gene, as found in previous studies. So far, a remarkably diverse array of 120 types of mutations had been identified, including 87 mutations leading to amino acid substitutions or stop codons, 30 nucleotide deletions or insertions including an insertion of IS6110 into the *pncA* gene, and 3 putative promoter mutations, in PZA-resistant strains from six independent studies (4, 6, 7, 10, 15, 16). Yet again, 17 new and diverse *pncA* mutations were found in this study. The highly diverse mutation profile in the *pncA* gene observed in PZA-resistant strains is unique among all drug resistance genes in *M. tuberculosis*. While the cause for this remarkable diversity of *pncA* mutations is unclear, it is possible that this could be due to adaptive mutagenesis or to a deficiency in DNA mismatch repair mechanisms in *M. tuberculosis* (12). Furthermore, the possibility that the *pncA* gene might be located in a hot spot of mutation in the genome cannot be ruled out. Another explanation relates to the non-essential nature of the *pncA* gene, as shown in this study with the spread of a PZA-mono-resistant strain, such that it can accumulate various mutations without affecting the viability of the organism. In contrast, in the case of RMP, streptomycin, kanamycin, EMB, and quinolone resistance, not all mutations in the target genes lead to a viable organism, such that only a limited array of mutations can be tolerated without losing the function of vital enzymes and fitness of the organism.

It is intriguing that we were unable to identify any mutations in the *pncA* gene or in the putative *pncA* promoter region in three PZA-resistant strains with negative PZase. This suggests that there could be a *pncA*-regulatory gene and that mutation of this gene could affect expression of *pncA*, thereby causing PZA resistance. Identification of this *pncA*-regulatory gene may be useful for designing a molecular test for better detection of PZA-resistant strains. In addition, we identified one PZase-positive, PZA-resistant strain, strain 9739, that did not have any *pncA* mutation. From the published reports on this topic (4, 6, 7, 10, 14, 15), it appears that such highly resistant *M. tuberculosis* strains with positive PZase activity and no *pncA* mutations are rare. However, this suggests that a new mecha-

TABLE 1. Characteristics of PZA-resistant clinical isolates of *M. tuberculosis*

Strain ^a	Resistance to other drugs ^c	PZA MIC (μg/ml)	PZase activity	<i>pncA</i> mutation(s) ^b	
				Nucleotide change(s)	Amino acid change(s)
9869	INH, RMP, SM	>300	–	–11 promoter mutation A→G	
10347	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift*
9131	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
11041	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
11135	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9721	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9769	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
11823	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9132	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9579	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10800	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10350	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
11243	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9811	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9004	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10348	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10257	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10003	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
11743	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
10611	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9155	Monoresistant	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
9953	INH, RMP, EMB	>300	–	C to A at 418, 8-bp deletion at 446	Arg140→Ser, frameshift
W39117	INH, RMP, EMB, Oflox	>900	–	A to G at 29	Gln10→Arg*
H3628	INH, RMP	>900	–	A to C at 35	Asp12→Ala
K10451	NT	>500	–	C to A at 137	Ala46→Glu
K10437	NT	>500	–	C to T at 137	Ala46→Val
F29684	INH, RMP, EMB	>900	–	C to T at 137	Ala46→Val
W5457	NT	>900	–	C to T at 137	Ala46→Val
A7259	INH, RMP, SM	>900	–	C to T at 137	Ala46→Val
A7183	INH, RMP, SM	>900	–	C to T at 137	Ala46→Val
8989	NT	>900	–	C to T at 137	Ala46→Val
K10552	NT	>100	–	A to C at 158	Asp53→Ala*
PZA-R9717	Monoresistant, in vitro mutant	>900	–	G to A at 203	Trp68→stop*
A7157	INH, RMP, SM	>900	–	T to G at 213	His71→Glu*
A7153	INH, RMP, EMB, SM	300	–	A to G at 245	His82→Arg*
K10400	NT	>500	–	T to G at 254	Leu85→Arg
T63168	INH, RMP, EMB, SM	>900	–	G to A at 289	Gly97→Ser*
M52997	INH, RMP, EMB, SM	>900	–	G to A at 289	Gly97→Ser
10426	INH, RMP, SM	>300	–	C to T at 401	Ala134→Val
K10551	NT	>500	–	G to C at 413	Cys138→Ser
K10428	NT	>500	–	G to A at 415	Val139→Met*
W76757	INH, RMP, EMB, SM	>300	–	A to C at 422	Gln141→Pro
K10447	NT	>500	–	T to G at 464	Val155→Gly
099-3-CIP8	RMP, Oflox	300	–	C to A at 503	Thr168→Asn*
10467	INH, RMP, SM	>300	–	G insertion at 52*	Frameshift
11627	SM	>300	–	A insertion at 193*	Frameshift
A7156	INH, RMP, SM	>900	–	A insertion at 193	Frameshift
K10429	NT	>500	–	G deletion at 301*	Frameshift
K10452	NT	>500	–	C deletion at 341*	Frameshift
H2374	RMP	>900	–	G insertion at 420*	Frameshift
H1033	INH, RMP, SM, Oflox	>900	–	G insertion at 420	Frameshift
K10436	NT	>100	–	GG insertion at 428*	Frameshift
T61823	INH, RMP, SM	>900	–	GG insertion at 420, T to G at 385	Val130→Gly*
11552	INH	>300	–	No mutation	
M49586	NT	>900	–	No mutation	
F57636	INH, RMP	>900	–	No mutation	
9739	NT	300	+	No mutation	
10274	NT	100	+	No mutation	
11830	NT	100	+	No mutation	

^a Strain designations with only numbers indicate strains from Quebec, Canada. Strain designations starting with K indicate strains from South Korea, and strain designations starting with other letters indicate U.S. strains.

^b Asterisks indicate new mutations not reported in previous studies (4, 6, 7, 10, 14, 15).

^c SM, streptomycin; Oflox, ofloxacin; NT, not tested.

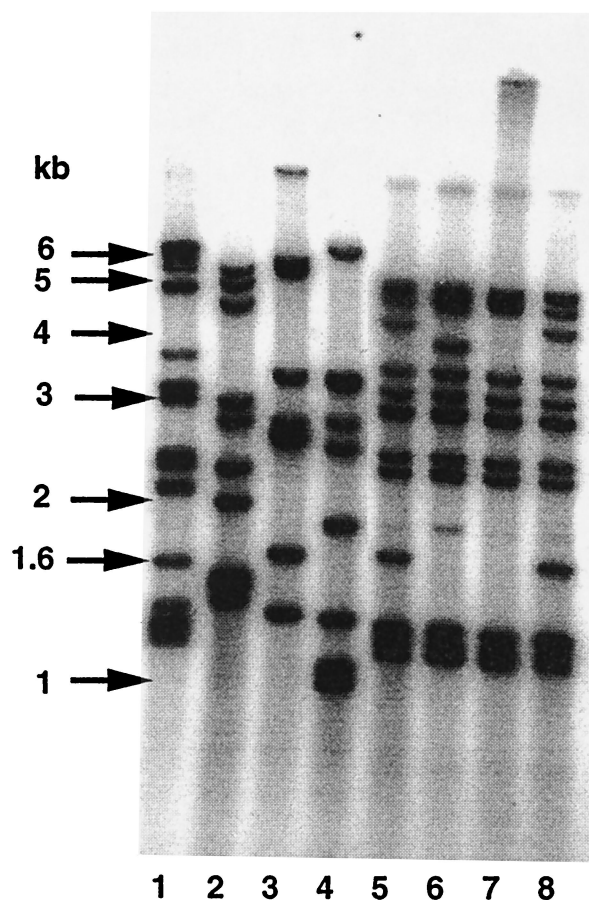


FIG. 1. IS6110 strain typing analysis of PZA-resistant *M. tuberculosis* strains. Lanes 1 and 2, strains T63168 and M52997, respectively, which share the same mutation of Gly97→Ser. Lanes 3 and 4, H2374 and H1033, respectively, which share the same mutation of nucleotide G insertion at position 420. Lanes 5 to 8, 10350, 10003, 9155, and 10257, which all share the same *pncA* mutation profile of an 8-bp deletion at nucleotide position 446 followed by Arg140→Ser.

nism of PZA resistance without affecting PZase activity or expression may exist. Mutations leading to modification or amplification of the POA target or to enhanced POA efflux could potentially cause PZA resistance. However, these alternative resistance mechanisms have yet to be identified. Strain 9739 may provide an opportunity to study alternative mechanisms of PZA resistance.

In this study, we found that two PZase-positive strains, initially identified as PZA-resistant based on the single-concentration test with 100 µg/ml by the BACTEC method, turned out to be susceptible to PZA (100 µg/ml) upon retesting. Sequence analysis showed that these strains did not have any *pncA* mutations. In view of this potential false-resistance problem, we would like to stress that the findings reported here support the previous suggestion (2) to use 300 instead of 100 µg of PZA per ml in a single-concentration test by the BACTEC method in pH 6.0 medium. The laboratory at the National Jewish Medical and Research Center in Denver, Colo., has been using this technique for many years with three PZA concentrations (100, 300, and 900 µg/ml) for a quantitative test to determine the MIC (1) and with 300 µg/ml for the single-concentration qualitative test (2). The 300-µg/ml PZA cutoff is less vulnerable to variations in the pH values that are likely to occur from different batches of the medium, as well as

more tolerant to slight increases in the inoculum size that could increase the MIC of PZA.

The transmission of a PZA-monoresistant TB strain in Quebec, Canada, is interesting. In the Province of Quebec, the average annual prevalence of PZA monoresistance for the past 5 years was 2.8% (L. Thibert, unpublished data). In this study, 21 PZA-monoresistant strains, which were isolated from different individual patients in 10 different geographic regions of Quebec, all had the same mutation profile—an 8-bp deletion at nucleotide position 446 followed by an amino acid substitution of Arg140→Ser. These patients are not known to have taken PZA monotherapy or PZA prophylaxis. IS6110 typing indicated that these strains are highly related and share almost identical IS6110 patterns. The finding of a slight difference in the IS6110 pattern (Fig. 1) among the 20 PZA-monoresistant strains and one MDR strain with the same mutation profile suggests that this clone has probably been in that area for some time. PZA monoresistance is unusual, as PZA is not used alone to treat TB, and often, if any single drug resistance occurs it is usually INH resistance. The finding that one MDR TB strain from Quebec also had the same type of mutation as the 20 PZA-monoresistant strains strongly suggests that the PZA-monoresistant strain emerged first and then was followed by an accumulation of other mutations leading to resistance to INH, RMP, and EMB. In this case, detection of *pncA* mutation helped to identify the transmission of a PZA-monoresistant TB strain. While INH-resistant, catalase-peroxidase-defective strains may have impaired ability to cause disease, it is clear from this study that PZA-resistant strains lacking the PZase enzyme are still fully capable of causing active disease. This is the first demonstration of an active transmission of TB due to a PZA-monoresistant strain by using a molecular epidemiology approach. It remains to be determined if PZA-monoresistant strains from other parts of Canada are related to those characterized in this study in terms of *pncA* mutation and IS6110 profile.

The increasing emergence and spread of drug-resistant *M. tuberculosis* worldwide pose serious threat to the control of TB. The rapid detection of drug-resistant strains represents an important part of the TB control strategy. While drug susceptibility testing could be reliably done for most TB drugs, PZA susceptibility testing is difficult and may produce inconsistent results and frequent false-resistance reports (3). Because of this, many clinical microbiology laboratories do not perform PZA susceptibility testing, and most drug resistance epidemiology surveys do not have PZA resistance data. Our finding of *pncA* mutations as a major mechanism of PZA resistance provides promise for developing a molecular test for rapid detection of PZA resistance based on detecting *pncA* mutations.

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