JCM
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Gene Sequencing for Routine Verification of Pyrazinamide Resistance in *Mycobacterium tuberculosis*: a Role for *pncA* but Not *rpsA*

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Pyrazinamide (PZA) is an important component of first-line therapy for the treatment of tuberculosis. Here, we evaluate targeted gene sequencing as a supplement to phenotypic PZA susceptibility testing of *Mycobacterium tuberculosis*. Routine sequencing of *pncA*, but not *rpsA*, is effective for verification of PZA susceptibility results.

Antimicrobial susceptibility testing is a key function of the mycobacteriology laboratory. Susceptibility results guide appropriate tuberculosis (TB) therapy and help prevent the emergence and spread of drug-resistant *Mycobacterium tuberculosis* strains. Pyrazinamide (PZA) is a front-line drug for the treatment of TB. Administered during the 2-month, intensive phase of the standard short-course regimen, PZA is effective primarily against slowly replicating bacilli and, thus, complements the activities of isoniazid (INH) and rifampin (RIF), which are bactericidal for rapidly replicating organisms.

PZA is a prodrug. Conversion to the active form, pyrazinoic acid (POA), is mediated by the pyrazinamidase (PZase) encoded by the *pncA* gene. It is well established that mutations in *pncA* can mediate PZA resistance by disrupting PZase activity and the accumulation of POA (6). However, some PZA-resistant (PZA^r) strains have wild-type *pncA* ($pncA^{WT}$) alleles. In such strains, resistance has been proposed to result from altered PZA uptake, increased POA efflux, or impaired POA binding to drug targets (7, 9). Recently, it has been demonstrated that POA binding to the 30S ribosomal protein S1 inhibits the trans-translation activity required for efficient protein synthesis (7). Mutations in *rpsA*, which encodes the S1 protein, result in altered POA binding and can mediate PZA resistance in $pncA^{WT}$ strains.

Despite the *in vivo* efficacy of PZA, *in vitro* susceptibility testing is challenging (2). PZase activity and the intracellular accumulation of POA increase with decreasing pH, but *Mycobacterium tuberculosis* viability decreases with decreasing pH. The Clinical and Laboratory Standards Institute (CLSI) recommends the Bactec 460TB radiometric system with Bactec 460TB PZA test medium (BD Diagnostics Systems, Sparks, MD) as the reference method for phenotypic PZA susceptibility testing (1, 4). However, the 460TB system has been discontinued and the 460TB PZA test medium is no longer being manufactured. Many laboratories, including Public Health Ontario (PHO), have adopted the Bactec MGIT 960 (BT960) platform for PZA testing. At our large public health laboratory, the switch to BT960-based testing was accompanied by an elevated incidence of false-positive results, defined as strains that were PZA^r by the BT960 method but PZA-susceptible (PZA^s) according to the reference 460TB method (3). To ensure accurate susceptibility results, confirmatory testing of potential PZA^r isolates is necessary. A phenotypic strategy, involving a second round of BT960-based testing, can be effective but does not resolve all cases (3, 8), and repeat testing requires an additional 5 to 7 days to complete (1). In contrast, confirmatory testing using molecular methods can be completed in less than 48 h. As such, we have investigated the utility of targeted gene sequencing for rapid verification of PZA^r results.

To assess the utility of sequencing, archived DNA from 141 previously characterized clinical isolates was examined. All strains were from the PHO strain collection and were originally isolated between 1999 and 2011. The *M. tuberculosis* complex designation, spoligotype, 12-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) pattern, and first-line drug susceptibility profiles of each isolate were determined previously as part of routine clinical testing (see Table S1 in the supplemental material). Review of the original PZA susceptibility results, performed using the 460TB method (1, 4), indicated that 77 isolates were PZA sensitive (PZA^s) and 64 were PZA resistant (PZA^r). For the current study, the *pncA* gene was amplified using primers pnc1 (5′-GGCGTCATGGACCCTATATC-3′) and pnc2 (5′-CAACAGTTCATCCCGGTTC-3′) (5). The 670-bp amplicon encompassed the complete *pncA* coding region and 80 bp of upstream DNA. Individual PCRs were prepared with HotStar Plus master mix (Qiagen, Toronto, Canada), 0.5 μM each primer, and 5 ng of template DNA. PCR was performed on a GS1 thermocycler (G-Storm Ltd.; Somerset, United Kingdom) under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min), and a final extension at 72°C for 10 min. DNA sequencing was performed via BigDye Terminator v3.1 cycle sequencing (Life Technologies, CA) with the Applied Biosystems 3730xl DNA analyzer (Life Technologies). All PZA^s strains were wild type for *pncA*, although a previously recognized variant allele (C195T, Ser65) was observed in a subset of East African Indian lineage isolates (10). In contrast, *pncA* mutations, including insertions, deletions, nonsynonymous changes, and promoter mutations, were present in 53 of 64 PZA^r isolates (Table 1; see also Table S1 in the supplemental material for additional

Received 5 March 2012 Returned for modification 16 April 2012
Accepted 7 August 2012
Published ahead of print 15 August 2012
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Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.00620-12

TABLE 1 Phenotypic and genotypic characteristics of pyrazinamide-resistant clinical isolates[a]

Isolate type	No. of isolates with each drug resistance phenotype[b]				Total no. of isolates
	PZA only	Poly[c]	MDR[d]	XDR[e]	
Mutant *pncA*	23	8	23	1	55
Promoter	2	1			3
Nonsynonymous	18	5	17	1	41
Insertion/deletion		2	6		8
Multiple mutations[f]	3				3
Wild-type *pncA*	5 (1)	1 (0)	3 (0)		9 (1)
All isolates					64 (56)

[a] Values in parentheses are the numbers of strains remaining after repeat testing of discordant isolates. A total of 8 strains were excluded. Five strains were reclassified as PZA sensitive, and three were not able to be retested.
[b] Drug susceptibility testing was performed on the 460TB (all 64 isolates) and BT960 (11 discordant isolates) platforms.
[c] Strains that are resistant to (at least) PZA and INH but not RIF.
[d] Strains that are resistant to (at least) PZA, INH, and RIF.
[e] XDR, extensively drug resistant.
[f] C418A and an 8-bp deletion were observed in all 3 isolates.

details). Eleven isolates were phenotypically PZA^r but genotypically *pncA*^{WT}. Of these discordant isolates, 5 were monoresistant PZA^r and 6 were resistant to multiple first-line drugs. Eight of the discordant isolates were successfully resurrected from frozen stocks, and first-line susceptibility testing was repeated using the BT960 method (3). In contrast to the original 460TB results, BT960-based testing indicated that 5 of these strains were PZA^s and, thus, was concordant with *pncA* sequencing. A cause for the discrepant BT960 and 460TB findings was not able to be determined, but the results indicate that the "gold standard" 460TB method is not infallible. Of the three remaining discordant isolates, one was PZA monoresistant and two were multidrug-resistant tuberculosis (MDR-TB) strains. Genomic DNA was extracted from the resurrected strains, and upon resequencing, *pncA* mutations were identified in both MDR-TB isolates. No mutations were found in the final isolate, and this one monoresistant strain remained phenotypically PZA^r and genotypically *pncA*^{WT}.

To examine the potential contribution of *rpsA* mutations to PZA resistance, 13 PZA^s strains and the 11 isolates initially considered discordant were analyzed (Table 2). To obtain full-length *rpsA* sequences, five overlapping amplicons were generated by PCR using the following primers: rspA-1F, 5′-ATGCCGAGTCCCACCGTC-3′; rspA-1R, 5′-ACCCTTGACGACCTCGATGA-3′; rspA-2F, 5′-AAACGCGCGCAGTACG-3′; rspA-2R, 5′-GTGACCTCGTCACCAACCTG-3′; rspA-3F, 5′-GACGGTCTGGTGCATGTCT-3′; rspA-3R, 5′-CTGCTTTTCGAATCCCTCAA-3′; rspA-4F, 5′-ATGGCTTGAGGGATTCGAAAA-3′; rspA-4R, 5′-AGCGCTGCCGGCGAGT-3′; rspA-M5F, 5′-AAACCAACGAATGGCTTGAG-3′; and rspA-M5R, 5′-CTACTGGCCGACGACTGAT-3′. PCR and sequencing conditions were identical to those described for *pncA* except that a longer extension time (72°C for 1.5 min) was used. A synonymous *rpsA* mutation (A636C, Arg212) was present in strains from both groups. Genotyping data indicate that this is not a lineage-specific mutation. One isolate also harbored a second synonymous change (G960A, Leu320). Nonsynonymous mutations were present in two strains. G1318A (Ala440Thr) was observed in the *Mycobacterium bovis* BCG reference strain and is conserved among publicly available genomes of *M. bovis* and *M. bovis* BCG. PZA resistance in *M. bovis* is a recognized phenomenon that is traditionally attributed to the *pncA* A169G (His57Asp) mutation. The impact of the RpsA Ala440Thr change remains to be determined, but an adjacent mutation, RpsA ΔAla438, has been characterized and found to impair POA binding to the 30S ribosomal protein S1 (7). Shi et al. (7) described two PZA^r/*pncA*^{WT} strains bearing dual RpsA Thr5Ser and Asp123Ala mutations. That combination was not observed in our study, but a similar mutation, *rpsA* A364G (Lys122Glu), was present in one PZA^s strain. Proteomic studies are required to determine if any of the Thr5Ser, Asp123Ala, or Lys122Glu mutations impact POA binding or simply represent regions of RpsA that tolerate amino acid substitutions. Our phenotypic findings suggest that RpsA Lys122Glu has no impact on PZA resistance, and thus, Asp123Ala may also be innocuous. However, sequence comparison indicates that Thr5Ser is present in the RpsA ortholog of *Mycobacterium avium.* Similarly, the RpsA ortholog of *Mycobacterium canettii* strain CIPT 140010059 exhibits 2 changes, Thr5Ala and Thr210Ala, relative to *M. tuberculosis* H37Rv. Strains of the *M. avium* complex and *M. canettii* isolates are considered PZA^r even though they are genotypically *pncA*^{WT} and exhibit PZase activity (9, 11). The Thr5Ser/Thr5Ala variants may explain this phenomenon.

Although *rpsA* may contribute to PZA resistance, *rpsA* sequencing required analysis of five overlapping PCR products, and

TABLE 2 Genotypic characterization of *rpsA* in clinical and reference isolates[a]

Characterization	No. of isolates with:					Total no. of isolates
		Synonymous change		Nonsynonymous change		
	Wild-type *rpsA*	A636C (Arg212)	G960A (Leu320)	A364G (Lys122Glu)	G1318A (Ala440Thr)	
460TB sensitive	11	1		1		13
460TB resistant and[b]:	7	4	1[c]			11
Not retested	1	2				3
BT960 sensitive	3	2	1[c]			5
BT960 resistant	3					3
M. bovis BCG					1	1

[a] The susceptibility profiles of eight strains were retested.
[b] Includes 11 discordant isolates initially considered PZA resistant and *pncA*^{WT}.
[c] Strain is a double mutant (A636C and G960A) and counted only once.

no phenotypically informative mutations were identified. In contrast, analysis of the single *pncA* amplicon uncovered mutations in all but four PZA^r isolates and no PZA^s strains. This indicates that, for *Mycobacterium tuberculosis*, DNA sequencing of *pncA* but not *rpsA* is a robust tool for routine and rapid verification of PZA susceptibility results.

ACKNOWLEDGMENTS

We acknowledge the excellent work of the Public Health Ontario TB and Mycobacteriology Laboratory (PHL-Toronto) staff responsible for the initial isolation, cultivation, and susceptibility testing of the clinical isolates used in the study.

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