Non-*pncA* Gene-Mutated but Pyrazinamide-Resistant *Mycobacterium tuberculosis*: Why Is That?

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ABSTRACT Pyrazinamide (PZA) is a key component for the effective treatment of drug-susceptible and PZA-susceptible multidrug-resistant (MDR^{PZA-S}) tuberculosis (TB). *pncA* gene mutations are usually detected in a clear majority (>90%) of PZA-resistant strains but obviously not in all. Rapid and reliable PZA drug susceptibility testing (DST) is critical whenever PZA is to be used in a treatment regimen, not least for the treatment of MDR^{PZA-S} TB. In this study, we selected 26 PZA-resistant isolates reported to carry a wild-type *pncA* gene. To confirm resistance, susceptibility testing was repeated using 100 mg/liter and 200 mg/liter PZA for all the 26 isolates and Sanger sequencing was repeated on the 18 isolates that remained PZA resistant. Apart from the eight isolates initially misclassified as PZA resistant, the retests identified three factors responsible for the phenotype-genotype discrepancy: *panD* or *rpsA* mutations identified by whole-genome sequencing (WGS) ($n = 7$), heteroresistance ($n = 8$), and mixed populations with *Mycobacterium avium* ($n = 3$). Additionally, we performed WGS on 400 PZA-susceptible isolates and 15 consecutive MDR^{PZA-R} clinical isolates. Of the 400 PZA-susceptible isolates, only 1 harbored a nonsynonymous *pncA* mutation (Thr87Met), whereas a nonsynonymous *rpsA* mutation was found in 17 isolates. None of these isolates carried a nonsynonymous *panD* mutation, while all 15 of the MDR^{PZA-R} isolates harbored a nonsynonymous *pncA* mutation. Our findings indicate that it is necessary to consider the occurrence of *panD* mutations in PZA-resistant isolates, as well as heteroresistance, for the development and evaluation of new molecular techniques to ensure high-quality DST performance. The identification of nonsynonymous *rpsA* mutations in both PZA-susceptible and PZA-resistant isolates also implies that further studies are needed in order to determine the role of *rpsA* in PZA resistance.

KEYWORDS pyrazinamide resistance, heteroresistance, *M. tuberculosis*, *pncA*, *rpsA*, *panD*, pyrazinamide, drug susceptibility testing

Pyrazinamide (PZA) is a first-line agent with a clear role in the treatment of both drug-susceptible tuberculosis (TB) and PZA-susceptible multidrug-resistant (MDR^{PZA-S}) TB.

The strong sterilizing effect of PZA on nonreplicating bacilli (persisters), in acidic environments such as in inflammatory tissues, has a unique role in enabling a shortened therapeutic regimen and cure of TB. Mutations in the *pncA* gene, encoding the pyrazinamidase that converts PZA into its active form, pyrazinoic acid, represent the major PZA resistance mechanism and are found in >90% of the PZA-resistant isolates (1, 2).

In contrast to the methods used with other first-line drugs, PZA drug susceptibility testing (DST) is not always performed due to the lack of appropriate techniques; e.g., PZA is not recommended for testing on solid media, which is the DST standard in many countries with a high incidence of tuberculosis. To date, the Bactec MGIT 960 platform (Becton Dickinson Biosciences, Sparks, MD) has been the only platform recommended

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for PZA DST by the WHO (3). There is a general view that DST is more complicated for PZA than for other anti-TB drugs, and some laboratories have experienced less reliability of PZA testing, usually due to pH-related difficulties. An increase of the pH in the test medium, caused by bacterial overinoculation, can give false resistance results (4, 5). Even when PZA testing is done, the use of proficiency testing for the quality assurance is implemented only rarely. In many regions, reliable DST results for PZA are usually unavailable, and the true level of PZA resistance is not known in many areas. Since 2013, the WHO Network of Supranational Reference Laboratories (SRLN) has offered for the first time yearly proficiency test panels for PZA also. The fact that no commercial molecular method is generally available for PZA testing, as it is for most other important first- and second-line drugs, makes quality assurance for phenotypic PZA DST even more justified. The lack of data, or at least of quality-assured data, on the PZA activity for clinical isolates is clearly problematic in a situation with increasing rates of MDR-TB. A reliable and affordable test method for the detection of PZA resistance is therefore greatly needed, particularly in settings where MDR tuberculosis is prevalent.

In many countries with a high incidence of tuberculosis where access to rapid MDR screening techniques is very limited, standard first-line treatment (including the use of PZA) may be administered to undiagnosed MDR^{PZA-S} patients for months while culture and DST on solid medium are ongoing, which increases the risk of developing MDR^{PZA-R}.

The typical interval of PZA resistance among MDR strains is between 40% and 60% (6–9), and the median prevalence of PZA resistance among MDR isolates in a meta-analysis published in 2011 was 51% (1). The large variation in the PZA resistance levels between regions probably illustrates differences in the PZA treatment strategies but also in laboratory performance with respect to correctly detecting PZA-resistant strains (10, 11).

Rapid molecular tools have successfully been developed for the screening of MDR and extensively drug-resistant (XDR) TB, for which most of the resistance is conferred by a limited number of predominant mutations (12, 13). Molecular assessment of PZA resistance is more complicated in this regard, since the resistance-conferring mutations are highly diverse and scattered throughout the *pncA* gene (14). Although PZA-resistant strains with no *pncA* gene mutations are rare, mutations in the *panD* (encoding the aspartate decarboxylase enzyme involved in the coenzyme A biosynthesis) and *rspA* (encoding ribosomal protein S1 involved in *trans*-translation) genes were recently reported to correlate with PZA resistance (15, 16).

Some reports, however, emphasize that some of the conventional molecular assays for routine determination of MDR may be less capable of detecting the important 1% critical proportion, which is the cutoff to define clinical drug resistance (17, 18). Heteroresistant isolates, cultured from patients infected with multiple *M. tuberculosis* strains (mixed infection) or from patients with a strain acquiring resistance during treatment, may therefore pose a challenge for the laboratory DST techniques.

In the present study, we were able to select 26 clinical isolates of *M. tuberculosis* carrying a wild-type (WT) *pncA* gene for an in-depth analysis of their phenotypically observed PZA resistance. Our findings may explain most of the phenotype-genotype discordances and should be considered in interpreting drug susceptibility test results as well as in evaluating and developing new DST techniques.

RESULTS

Bactec MGIT drug susceptibility testing. As illustrated in Table 1, the phenotypic Bactec MGIT DST confirmed PZA resistance in 18 of the 26 non-*pncA*-mutated PZA-resistant isolates. Eight isolates were, however, clearly susceptible to the critical test concentration of 100 mg/liter PZA and were excluded from further analysis. None of the non-*pncA*-mutated PZA-resistant isolates were inhibited at 100 mg/liter PZA, but two (both harboring the Val260Ile mutation in the *rpsA* gene) did not grow at 200 mg/liter PZA (Table 1). All 400 of the PZA-susceptible consecutive isolates were inhibited at 100 mg/liter PZA, but none of the 15 MDR^{PZA-R} isolates were.

TABLE 1 Extended analysis of the 26 *M. tuberculosis* isolates selected as PZA resistant with wild-type *pncA* gene^a

Parameter and strain ID	1st DST result			2nd DST Bactec result (PZA [100/200 mg/liter])	Result of Sanger sequencing of <i>pncA</i> gene		WGS result	Lineage according to spoligotyping	
	INH	RIF	EMB		PZA	1st DST			MGIT PZA tube
False resistance									
1	S	S	S	R	S/S	WT	ND		
2	S	S	S	R	S/S	WT	ND		
3	S	S	S	R	S/S	WT	ND		
4	S	S	S	R	S/S	WT	ND		
5	R	S	R	R	S/S	WT	ND		
6	S	S	S	R	S/S	WT	ND		
7	ND	ND	ND	R	S/S	WT	ND		
8	S	R	R	R	S/S	WT	ND		
Heteroresistance									
9	R	R	R	R	R/R	WT	Thr142Lys		
10	R	R	S	R	R/R	WT	Leu182Ser		
11	R	R	S	R	R/R	WT	InDel codon 131		
12	ND	ND	ND	R	R/R	WT/Trp68Gly	Trp68Gly		
13	S	S	S	R	R/R	WT/Gly97Ser	Gly97Ser		
14	ND	ND	ND	R	R/R	WT	del nt –5 to nt 7		
15	S	S	S	R	R/R	WT/Ser65Ser	del promoter + del <i>pncA^b</i>		
16	R	R	R	R	R/R	WT	InDel codon 136		
Alternative mechanism									
17	S	S	S	R	R/R	WT	Ile115Thr	WT	LAM
18	S	S	S	R	R/R	WT	WT	Ile55Val + Val260Ile	EAI
19	S	R	S	R	R/S (low level)	WT	WT	Val260Ile	EAI
20	S	S	S	R	R/S (low level)	WT	WT	Val260Ile	EAI
21	S	S	S	R	R/R	WT	Ile49Val	WT	EAI
22	S	S	S	R	R/R	WT	Ile49Val	WT	EAI
23	S	S	S	R	R/R	WT	Ile115Thr	WT	LAM
<i>M. tuberculosis</i> + <i>M. avium</i>									
24	R	R	S	R	R/R	WT	ND		
25	ND	ND	ND	R	R/R	WT	ND		
26	S	S	S	R	R/R	WT	ND		

^aISO, isoniazid (0.1 mg/liter); RIF, rifampin (1 mg/liter); EMB, ethambutol (5 mg/liter); PZA, pyrazinamide (100 mg/liter); S, susceptible; R, resistant; ND, not determined; del, deletion; nt, nucleotide. The most important findings are boldfaced.

^bFailure of Sanger sequencing due to a deleted primer sequence in *pncA*; genotype identified by WGS.

TABLE 2 Nonsynonymous *pncA*, *rpsA*, and *panD* mutations in consecutive clinical isolates

Mutation	No. of isolates	
	PZA susceptible (<i>n</i> = 400)	MDR ^{PZA-R} (<i>n</i> = 15)
<i>pncA</i>	1	15
<i>panD</i>	0	0
<i>rpsA</i>	17	1
<i>rpsA</i> Asp37Ala	1	0
<i>rpsA</i> Lys45Gln	1	0
<i>rpsA</i> Lys45Glu	0	1
<i>rpsA</i> Glu197Lys	1	0
<i>rpsA</i> Val254Gly	1	0
<i>rpsA</i> Val260Ile	5	0
<i>rpsA</i> Asp268Asn	1	0
<i>rpsA</i> Glu405Lys	1	0
<i>rpsA</i> Met432Thr	6	0

Sanger sequencing. For 15 of the 18 *pncA* wild-type PZA-resistant isolates, sequencing of the *pncA* gene was repeated on growth from the Bactec MGIT PZA-containing tubes (Table 1). The other three isolates proved to be a mixture with *Mycobacterium avium* (detected by whole-genome sequencing [WGS] and subsequent analysis of the *rrs* gene) and were excluded from further analysis. This time, seven strains exhibited *pncA* gene polymorphisms identified as nonsynonymous mutations (*n* = 4), insertions (*n* = 2), and deletions (*n* = 1) and were therefore classified as heteroresistant. For one strain, *pncA* gene amplification failed using the standard Sanger protocol, whereas seven strains remained nonmutated in the *pncA* gene.

Whole-genome sequencing (WGS). To obtain the *pncA* genotype for the sample lacking a Sanger result and to analyze alternative target genes in the seven nonheteroresistant PZA-resistant strains carrying wild-type *pncA*, WGS was performed. In the first case (strain identification no. [ID] 15; Table 1), the WGS analysis revealed a large (1,193-bp) deletion covering the putative *pncA* promoter as well as the first 158 bp of the *pncA* gene, presumably explaining both the Sanger failure and the PZA resistance phenotype.

WGS showed that all seven *pncA* WT PZA-resistant strains harbored a nonsynonymous mutation in either *panD* or *rpsA*. The *panD* mutations Ile49Val and Ile115Thr were identified in two strains each, whereas the three remaining strains carried the Val260Ile mutation in *rpsA* (in combination with Ile55Val in strain ID 15) (Table 1). Additionally, a whole-genome-based SNP analysis was applied in order to rule out patient-to-patient transmission within the three pairs which had identical *pncA*, *panD*, and *rpsA* sequences (data not shown).

To further analyze the importance of the identified *panD* and *rpsA* mutations, we screened another 400 consecutive PZA-susceptible isolates and 15 consecutive MDR^{PZA-R} isolates for mutations in these genes (all being clinical isolates from Sweden).

Among the 400 PZA-susceptible consecutive clinical isolates, WGS identified a nonsynonymous *pncA* mutation (Thr87Met) in 1 isolate whereas 17 isolates harbored a nonsynonymous mutation in *rpsA* (Table 2). Overall, 8 different nonsynonymous *rpsA* mutations were identified and 1 of them, Val260Ile, was also detected in three of the non-*pncA*-mutated isolates with resistance to PZA. Spoligotyping revealed that all isolates harboring this mutation, including both the PZA-susceptible and the PZA-resistant isolates, belonged to the East African Indian lineage. Correspondingly, none of the 400 isolates carried a mutation in *panD* (a synonymous mutation, Leu87Leu, was found in 3 of these isolates, however). All 15 consecutive MDR^{PZA-R} isolates were mutated in the *pncA* gene, with one strain also having the *rpsA* Lys45Glu mutation; on the other hand, no *panD* gene mutations were found in this group of strains (Table 2). A few synonymous *rpsA* mutations were also found among both the PZA-resistant and the PZA-susceptible isolates (data not shown).

DISCUSSION

Twenty-six clinical isolates of *M. tuberculosis* previously found to be phenotypically PZA resistant but lacking *pncA* gene mutations were selected in this study for a deep analysis of the discrepancy between the phenotypic and molecular DST results. We were able to identify the cause of the disagreements for all 26 samples; eight strains were found to be of a heteroresistance genotype, seven were mutated in either the *panD* or *rpsA* gene, three were mixed with *M. avium*, and eight were misclassified as PZA resistant in the initial DST, which may reflect problems with the phenotypic PZA analysis in some laboratories. These findings clearly show that the biological complexity of the strains needs to be managed by the laboratory drug susceptibility testing techniques in order to produce reliable results. The true magnitude of *panD* and *rpsA* mutations among clinical isolates with PZA resistance is difficult to determine since most laboratories do not routinely analyze these genes. With the expanding use of WGS, such data will become available to a much greater extent than has been the case to date. Among the 50 PZA-resistant *M. tuberculosis* clinical isolates detected in Sweden during 2015 to 2016 (28 of which were also MDR), all but 4 (of which 2 carried a *rpsA* mutation instead and 2 had a *panD* mutation [strain IDs 19 to 22 in Table 1]) were mutated in *pncA* (data not shown). A recent study also showed that among 174 *M. tuberculosis* H37Rv PZA-resistant mutants generated *in vitro*, 97% were *pncA* mutated, none were found to have mutations in *rpsA*, and only 3% harbored a *panD* mutation (19). Another study from the national TB referral center of China reported 0% *panD*, 7% *rpsA*, and 79% *pncA* gene mutations among 82 MDR^{PZA-R} clinical isolates, but *rpsA* mutations were also found in 10% of the PZA-susceptible isolates (20). Moreover, the *panD* mutation Ile49Val identified in two of our PZA-resistant strains has previously been detected in PZA-resistant mutants derived from *M. tuberculosis* strain H37Rv but was detected then as a double mutation together with His21Arg (19).

It is important to keep in mind that no assessment of heteroresistance is done in most studies, which can lead to underreporting of genotypic resistance among phenotypically resistant isolates. In this study, the presence of *panD* and *rpsA* mutations was related to PZA resistance in 7 of the 26 *pncA* wild-type strains whereas mutations in *pncA* conferred resistance in all 15 of the consecutive MDR^{PZA-R} isolates. Only one nonsynonymous *pncA* mutation (Thr87Met), classified as a mutation not causing resistance, was detected among the 400 PZA-susceptible isolates, demonstrating a strong correlation between *pncA* wild-type sequence and PZA susceptibility. The eight nonsynonymous mutations in *rpsA* distributed among 17 of the 400 PZA-susceptible consecutive isolates make this gene a more dubious predictor of PZA resistance. Interestingly, Val260Ile was the only *rpsA* mutation represented among both PZA-susceptible isolates ($n = 5$) and *pncA* wild-type PZA-resistant strains ($n = 2$), both of which were inhibited at 200 mg/liter PZA, indicating an association with low-level resistance. All isolates carrying this mutation were classified as East African Indian (EAI) by spoligotyping, and this finding may suggest that Val260Ile is in fact a phylogenetic marker for this particular lineage; on the other hand, however, less than 20% of the PZA-susceptible EAI isolates analyzed in this study harbored this mutation. A comprehensive review that was recently published by Njire et al. showed that further studies are needed to ascertain the role of *panD* and, especially, *rpsA* gene mutations in PZA-resistant tuberculosis (21).

In many areas with a high burden of MDR-TB, the absence of molecular techniques for early MDR detection and of information on the susceptibility to PZA is clearly problematic and reliable data that could predict the clinical usefulness of adding PZA to the therapeutic regimen are very rare. Consequently, PZA may be administered to undiagnosed MDR^{PZA-S} TB patients during long periods, increasing the risk of treatment failure and of development and transmission of MDR^{PZA-R} TB. Therefore, rapid and reliable PZA DST is critical whenever PZA is to be used in the regimen, not least for the treatment of PZA-susceptible MDR-TB. Various DST techniques are vitiated as a consequence of their specific limitations with respect to accurate determination of drug

resistance. For example, Bactec MGIT 960 can produce false PZA resistance results if the bacterial inoculums are not carefully prepared (5) and the current critical breakpoint may also classify a few strains as borderline resistant (22). On the other hand, molecular assays may not be sensitive enough to detect drug-resistant subpopulations (heteroresistance) at the critical 1% proportion level (10% for PZA) defining clinical resistance (17, 18, 23). The magnitude of mixed and polyclonal infections among TB patients is relatively unknown, although recent studies clearly showed that heteroresistance occurs frequently (~20% to 45%) in specific TB populations and that it may also have a negative impact on the treatment outcome (24–26).

Our findings illustrate how the biological complexity of *M. tuberculosis* strains can affect the reliability of the (PZA) DST and point out causes that may go undetected in the routine analysis. From this analysis, we suggest that DST of PZA, in conformity with the testing of other important first- and second-line drugs, always should be quality assured and that assessment of heteroresistance and screening of the *panD* and *rpsA* gene should be performed on PZA-resistant strains without *pncA* mutations.

MATERIALS AND METHODS

***M. tuberculosis* isolates.** The Supranational Reference Laboratory for Tuberculosis (SRL) at the Public Health Agency of Sweden (PHAS) receives all *M. tuberculosis* strains isolated from the five Swedish clinical TB laboratories to perform epidemiological typing. All isolates are stored at -80°C in the national strain collection at the agency. *pncA* gene sequencing has been used since 2012 for various studies and since 2013 also routinely for all Swedish MDR and mono-PZA-resistant clinical isolates. After reviewing the DST data files, we found a total of 26 clinical isolates with the wild-type (WT) *pncA* gene that had previously been reported to be PZA-resistant *M. tuberculosis*. In a comparative reference study panel, we included 400 PZA-susceptible strains and 15 consecutive MDR^{PZA-R} strains isolated from the same number of patients during 2016. In addition, spoligotyping was performed as previously described and genotypes were assigned through SITVIT WEB (27, 28).

Bactec MGIT PZA susceptibility testing. The phenotypic PZA susceptibility of the 400 PZA-susceptible consecutive clinical isolates was previously routinely determined with the Bactec MGIT 960 at any of the five clinical TB laboratories in Sweden. At the Public Health Agency, the Bactec MGIT 960 DST was repeated to confirm the PZA resistance of the 26 *pncA* WT PZA-resistant clinical isolates as well as for the 15 consecutive MDR^{PZA-R} isolates using a Becton Dickinson PZA kit.

The DST inoculum was prepared from bacterial growth on Löwenstein-Jensen egg medium incubated at 37°C for 21 days. Briefly, two 1- μl loops of bacteria were suspended in 3 ml of phosphate-buffered saline (PBS) in a small glass tube with glass beads. Homogenization of the bacterial suspension was performed using an ultrasound water bath to disperse clumps. Thereafter, the suspension was left to sediment for 20 min and the upper 2-ml volume was transferred to a new tube and left to sediment for another 15 min. Prior to inoculation of the Bactec MGIT PZA medium culture tubes (pH 5.9), the bacterial suspension was adjusted to a McFarland turbidity of 0.5 and diluted in PBS per the PZA test protocol from the manufacturer. The susceptibility of bacteria to the critical concentration of 100 mg/liter PZA as well as to 200 mg/liter PZA was assessed using a Becton Dickinson PZA kit.

Following the instructions from the manufacturer, susceptibility to PZA was defined as a growth unit (GU) level of <100 of the culture tube containing 100 mg/liter PZA at the time that the 1:10-diluted drug-free control had reached a GU level of 400.

Strains were defined as showing low-level resistance when the GU value of the 100 mg/liter PZA tube was >100 but the GU value of the 200 mg/liter PZA tube was <100 at the time that the GU value for the 1:10-diluted drug-free control was 400.

Sequencing of the *pncA* gene. Sequencing of the *pncA* gene was performed to detect mutations related to PZA resistance and according to our earlier described protocol (29). Briefly, the 561-bp-long *pncA* gene was amplified together with approximately 200 nucleotides upstream and downstream of the gene using *pncA_F3* (AAGGCCGCGATGACACCTCT) and *pncA_R4* (GTGTCGTAGAAGCGGCCGAT) as primers. ExoSAP-IT (Affymetrix, USA) was used to purify the PCR products, and the sequencing reactions were performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) together with the *pncA_F3*, *pncA_R4*, *pncA_P3-F* (ATCAGCGACTACCTGGCCGA), and *pncA_P4-R* (GATTGCCGACGTGTC CAGAC) primers. The sequencing reaction mixtures were purified with a BigDye Xterminator purification kit (Applied Biosystems, USA), and the sequencing data obtained from a model 3500xL genetic analyzer (Applied Biosystems, USA) were subsequently analyzed using CLC Main Workbench and the *pncA* sequence from H37Rv ATCC 25618 as the reference.

Detection of heteroresistance. To detect possible PZA-resistant subpopulations (which can be missed or overlooked by the conventional molecular techniques) in the non-*pncA*-mutated PZA-resistant isolates, standard Sanger sequencing was repeated directly on the selective growth from the MGIT tube containing PZA.

Whole-genome sequencing (WGS). All 400 of the PZA-susceptible isolates and the 15 consecutive MDR^{PZA-R} clinical isolates as well as the *pncA* WT PZA-resistant isolates with no heteroresistance genotype were further analyzed by WGS in order to screen for mutations in alternative target genes *panD* and *rpsA* (*pncA* and *rrs* were also included in the reference set, *pncA* to confirm the previous Sanger result and *rrs*

to detect possible contaminants in the bacterial isolates). Briefly, DNA extracted from LJ cultures (QIAamp DNA minikit; Qiagen, Hilden, Germany) was sequenced using IonTorrent PGM technology (Thermo Fisher Scientific Inc., Waltham, MA, USA). Library and emulsion PCR assays were performed according to the manufacturer's instructions. Mapping to reference sequences, derived from the *M. tuberculosis* H37Rv reference genome (GenBank accession no. NC_000962.3), and variant calling were performed in CLC Genomics Workbench 8 (Qiagen, Hilden, Germany).

Accession number(s). Raw reads from the seven PZA-resistant isolates carrying wild-type *pncA* were submitted to the Sequence Read Archive (PRJNA379513).

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We declare that we have no conflicts of interest.

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