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Improved Detection by Next-Generation Sequencing of Pyrazinamide Resistance in *Mycobacterium tuberculosis* Isolates

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The technical limitations of common tests used for detecting pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis* isolates pose challenges for comprehensive and accurate descriptions of drug resistance in patients with multidrug-resistant tuberculosis (MDR-TB). In this study, a 606-bp fragment (comprising the *pncA* coding region plus the promoter) was sequenced using Ion Torrent next-generation sequencing (NGS) to detect associated PZA resistance mutations in 88 recultured MDR-TB isolates from an archived series collected in 2001. These 88 isolates were previously Sanger sequenced, with 55 (61%) designated as carrying the wild-type *pncA* gene and 33 (37%) showing mutations. PZA susceptibility of the isolates was also determined using the Bactec 460 TB system and the Wayne test. In this study, isolates were recultured and susceptibility testing was performed in Bactec 960 MGIT. Concordance between NGS and MGIT results was 93% ($n = 88$), and concordance values between the Bactec 460, the Wayne test, or *pncA* gene Sanger sequencing and NGS results were 82% ($n = 88$), 83% ($n = 88$), and 89% ($n = 88$), respectively. NGS confirmed the majority of *pncA* mutations detected by Sanger sequencing but revealed several new and mixed-strain mutations that resolved discordancy in other phenotypic results. Importantly, in 53% (18/34) of these isolates, *pncA* mutations were located in the 151 to 360 region and warrant further exploration. In these isolates, with their known resistance to rifampin, NGS of *pncA* improved PZA resistance detection sensitivity to 97% and specificity to 94% using NGS as the gold standard and helped to resolve discordant results from conventional methodologies.

Pyrazinamide (PZA) is a cornerstone first-line antituberculosis compound that is also commonly used in the second-line therapeutic treatment of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) infection in humans. The drug is a structural analogue of nicotinamide, requiring conversion into its active form, i.e., pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase (PZase) (1, 2). PZase is encoded by the *M. tuberculosis pncA* gene (561 bp) (2). It has been postulated that the mechanism of action of PZA is through pyrazinoic acid, causing disruption of bacterial membrane-mediated energetics and ultimately causing inhibition of membrane transport (3). The contribution of PZA to the killing of *M. tuberculosis* as part of a multidrug regimen for the treatment of tuberculosis (TB) is considerable, and its inclusion with rifampin in anti-TB therapeutic regimens has resulted in the significant shortening of treatment duration from 18 to 6 months (3). The drug is known to specifically target semidormant bacteria that are not killed by other antituberculosis drugs (4, 5).

Mutations in the *pncA* gene are associated with phenotypically pyrazinamide-resistant isolates of *M. tuberculosis* (2, 6, 7). Such mutations can occur in the promoter or coding regions and result in amino acid substitutions, frameshifts, or stop codon mutations. Furthermore, phenotypic resistance has been reported in the literature for isolates containing the wild-type gene, suggesting the existence of a possible additional gene location involved in pyrazinamide resistance (2, 8).

Inclusion of pyrazinamide in second-line treatment regimens for multidrug-resistant (MDR) cases of TB is rarely based on the PZA susceptibility status of clinical isolates mainly because phenotypic assays are difficult to perform. This is primarily due to the drug being active only in an acid medium, posing challenges to drug susceptibility testing (DST) in the clinical laboratory (4, 9). Phenotypic methods for testing the susceptibility of *M. tuberculosis* to pyrazinamide and specifically the use of the Bactec 960 mycobacterial growth indicator tube (MGIT) liquid culture test system is currently regarded as the gold standard for determination of PZA resistance (10). Recent guidelines, however, recommend the use of the PZA test kit for MGIT 960 (11).

Two recent studies in South Africa revealed that 52% of all multidrug-resistant *M. tuberculosis* (MDR-TB) isolates carry resistance-conferring *pncA* gene mutations (5, 12). In addition, the Bactec 460 radiometric culture assay and the Wayne enzymatic assay, which measure loss of pyrazinamidase activity as a measure of resistance, indicated marked discrepancies to Sanger sequencing in identifying resistance to PZA (12).

Currently, there is a clear need to more accurately define the nature of PZA resistance at the genotypic level and, in particular, to assess the potential impact of PZA resistance on treatment outcomes in patients as judged by currently available and emerging technologies. In this study, using Ion Torrent next-generation sequencing (NGS) and the Bactec 960 MGIT liquid culture assay, we

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reinvestigated a collection of *M. tuberculosis* isolates previously studied and characterized for PZA resistance using the Bactec 460 radiometric assay, the Wayne test, and Sanger sequencing (12). We also aimed to detect heteroresistance using NGS, i.e., the coexistence of susceptible and resistant strains in the same isolate, as a possible cause of discrepant results between tests.

MATERIALS AND METHODS

Description of isolates. A national survey of drug resistance in *M. tuberculosis* isolates from pulmonary cases of TB was conducted between 2001 and 2002 in South Africa (12). All isolates from a survey collection of 5,866 isolates were tested for susceptibility to rifampin (RIF), isoniazid (INH), streptomycin (STM), and ethambutol (EMB). Of these, 179 were labeled as multidrug resistant (9.9% of these were designated resistant to all four test drugs). Specimens were collected from South African patients across eight of the nine provinces in the country. The 179 MDR-TB isolates were stored in Greaves medium in a −80°C freezer and were uninterrupted for 12 years since collection. Of these isolates, 130 were available for further investigation and 88 were successfully recultured after removal from storage. Frozen cultures were thawed for 24 h, and 200 μl of each original culture suspension was inoculated into a Bactec 960 MGIT liquid culture medium tube. Only one passage was performed between Bactec 460 and MGIT 960 testing from the original frozen cultures. The H37Rv reference strain was included as a positive control in this study. Pyrazinamide susceptibility of 86 of the 88 recultured isolates was previously determined using the Bactec 460 TB system, while all 88 isolates had previously been tested using the Wayne test and Sanger sequencing (10). In this study, we reinvestigated PZA susceptibility using the Bactec 960 MGIT PZA test kit as per the manufacturer's instruction, and we also applied NGS to the *pncA* gene for detection of confirmatory mutations.

MGIT-positive isolates were checked for contamination by streaking on blood agar plates and incubating for 24 h at 37°C. Following subculture, 0.5 ml MGIT medium containing *M. tuberculosis* isolates was transferred to 1.5 ml PrimeStore molecular transport medium (MTM; Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) and shipped at ambient temperature to Longhorn Vaccines & Diagnostics for whole-gene sequencing analysis using the Ion Torrent personal genome machine (Life Technologies, Foster City, CA, USA). PrimeStore MTM preserves sample nucleic acid integrity at ambient temperature for subsequent molecular analysis (13).

Phenotypic drug susceptibility testing. Pyrazinamide susceptibility testing, using the Bactec MGIT 960 system in the Becton, Dickinson commercial PZA kit, was performed per the manufacturer's instruction. Isolates of *M. tuberculosis* in MGIT were used as test inocula. A drug-free control sample was inoculated with a 1:10 dilution of inoculum. The PZA test sample contained 500 μl inoculum and 100 μl PZA. Assay tubes were monitored using a Bactec MGIT 960 instrument until the control assay tested positive. The PZA test was considered resistant or susceptible based on growth unit (GU) values of ≥100 GU or <100 GU, respectively.

Determination of genotypic resistance patterns. (i) DNA extraction. Mycobacterial DNA was extracted from all 90 isolates using PrimeXtract (Longhorn Vaccines & Diagnostics, San Antonio, TX, USA). Briefly, 200 μl 100% (vol/vol) ethanol, 200 μl lysis buffer, and 200 μl *Mycobacterium tuberculosis* inoculum were transferred to a 1.5-ml microcentrifuge tube. After thorough mixing and subsequent centrifugation, the entire supernatant was applied to a microextraction column, was centrifuged for 1 min at 13,000 rpm, and the flowthrough material was discarded. Wash buffer (200 μl) was applied to the extraction column and was centrifuged for 1 min at 13,000 rpm, followed by further addition of wash buffer (200 μl) to the extraction column and with subsequent centrifuging in similar fashion as described above, discarding the flowthrough material. Total *M. tuberculosis* DNA was eluted by 1 min of centrifugation at maximum speed using 50 μl of preheated (~75°C) elution solution. Total *M. tuberculosis* DNA was stored at −20°C until used.

(ii) Amplification and sequencing of the amplified *pncA* gene. The *pncA* forward/reverse primers included pncAF1 (5′-CGGATTTGTCGCTCACTAC-3′) and pncAR1 (5′-GCCGGAGACGATATCCAGAT-3′), comprising the full gene and also the *pncA* promoter region. The expected size of the *pncA* amplicon was 960 bases (14). PCRs were performed in a total volume of 50 μl, and the PCR mixture consisted of 5 μl of 10× buffer plus $MgCl_2$ (Longhorn Vaccines & Diagnostics, San Antonio, TX, USA), 2 μl of 20 μM forward and reverse *pncA* primers (Integrated DNA Technologies, Coralville, IA, USA), 0.5 μl Platinum *Taq* enzyme (Life Technologies, Grand Island, NY, USA), 35.5 μl nuclease-free water (Integrated DNA Technologies), and 5 μl extracted DNA. Amplification was performed on an ABI 9700 thermocycler (Life Technologies, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 15 s, and extension at 72°C for 1 min with a final extension cycle at 72°C for 5 min. PCR products were analyzed by 1% (wt/vol) agarose gel electrophoresis (Phoenix Research Products, Candler, NC, USA) and were viewed using a UV Transilluminator (Spectroline, Westberry, NY, USA).

(iii) Next-generation sequencing. Whole-gene next-generation Ion Torrent sequencing was performed using a novel but standardized *M. tuberculosis* protocol (14). All PCR products were subjected to full-gene *pncA* sequencing. Library preparation was carried out using the Ion Xpress plus fragment library kit (catalog no. 4471269) for fragmentation and adaptor ligation with an approximately 10- to 100-ng DNA amplicon. The DNA amplicon was sheared using the Ion Xpress Shearing II kit modified as follows: 21.5 μl PCR template, 2.5 μl 10× shear buffer, and 1.0 μl shearing enzymes. Adapter ligation, nick repair, and amplification (8 cycles) were all performed per the manufacturer's protocol. The prepared library was bar-coded using the Ion Xpress barcode adaptors 1-96 kit (catalog no. 4474517). The amount of library nucleic acid required for template preparation was made equal using the Ion Library Equalizer kit (Catalog no. 4482298) per the manufacturer's instruction. Emulsion PCR and recovery steps were carried out using the Ion PGM Template OT2 400 kit (catalog no. 4479878) according to the manufacturer's instructions. Ion Sphere particle quality assessment was carried out using the Ion Sphere quality control kit. Bioinformatics read assembly and multiple-sequence alignment were performed using SeqMan NGen (v4) and Lasergene (v10) core suite (DNAStar, Madison, WI, USA).

RESULTS

Bactec 960 MGIT. Of 88 tests giving susceptibility results, 60 (68%) were designated susceptible to pyrazinamide and 28 (32%) were designated resistant.

Ion Torrent next-generation sequencing. Results are summarized in Table 1. Of the 88 isolates sequenced, 55 (62%) were wild type and 33 (38%) had mutations representing substitutions, insertions/deletions, and stop codons. Ion Torrent detected seven heteroresistant mutations in seven isolates (L159P/L, S59P/S, R154G/R, D12G/D, D35Y/D, L35R/L, and T135P/T). Three isolates carried mutations at positions (T −10 C and T −12 C) of the *pncA* gene promoter by Ion Torrent NGS (Table 1). Ten substitutions were found in 12 isolates (C14R, S164P, L35R, L515S, H71Y, D12G, D154G, A102V, D8Y, and A79V). Four insertions/deletions were detected in five isolates in this study (insertion of T at position 360 [Ins Tpos360], Ins Gpos35, Ins Gpos315, and deletion of T at position 515 [Del Tpos515]). Only one stop codon was found in one isolate (K96STOP). In this study, 12 isolates had mutations within the 3 to 17 region, 3 isolates had mutations within the 61 to 85 region, and only 1 isolate had a mutation within the 132 to 142 region. The remaining isolates had mutations in the 151 to 360 region.

Historical PZA resistance results from other tests. For the 88 isolates included in this study, 46 (52%) were PZA susceptible by

TABLE 1 Bactec MGIT 960 PZA susceptibility test outcomes compared with Ion Torrent *pncA* whole-gene sequencing results

MGIT 960 results[a]	Ion Torrent sequencing results and description of mutations found	No. of strains
S	WT[b]	53
S	A102V	1
R	C14R	2
R	D12G	2
R	D12G/D (mixed)	3
R	D35Y/D (mixed), L35R/L (mixed)	1
R	D8N	1
R	D8Y, L35R	1
R	Extra G at nucleotide position 315	2
R	D8N	1
R	D8Y, L35R	1
R	Extra G at nucleotide position 35	2
R	G97C	1
R	H71Y	1
R	Deletion of T at position 515	1
R	K96STOP	1
R	L151S	2
R	L159P/L (mixed), T135P/T (mixed)	1
S	L35R	1
S	D12G	1
S	R154G	1
S	Promoter mutation T −10 C	1
R	Promoter mutation T −12 C	2
R	R154G	1
S	R154G/R (mixed)	1
R	S164P	1
R	S59P/S (mixed)	1
R	A 79 V, extra T at position 360	1

[a] S, susceptible; R, resistant.
[b] WT, wild type.

Bactec 460, 40 (46%) were resistant, and 2 (2%) were borderline (counted as susceptible in Table 2). Using the Wayne enzymatic assay, 59 (67%) isolates were susceptible and 29 (33%) were resistant. Using Sanger sequencing, 60 (68%) were susceptible and 28 (32%) were resistant.

Concordance/discordance between phenotypic tests and Ion Torrent NGS results. Bactec MGIT 960 results concurred with Ion Torrent 960 results in 93% of the 88 isolates (53 susceptible, 29 resistant, and 6 discordant). Sensitivity and specificity of MGIT 960 for detecting PZA resistance was 82% and 96%, respectively, using Ion Torrent as the gold standard. Of the 8 discordant results, 6 isolates were susceptible by MGIT 960, but mutations were detected by the Ion Torrent sequencing assay; two isolates were resistant by MGIT 960 but sequenced as wild type by Ion Torrent (Table 2). A comparison of Ion Torrent NGS results to earlier results from the older assays revealed the confirmation of 86% of

TABLE 2 Summary of PZA drug susceptibility test outcomes comparing different phenotypic and genotypic test methods

	No. (%) of sensitive or resistant isolates[b] using:				
Susceptibility[a]	MGIT 960	Bactec 460	Wayne test	Sanger	Ion Torrent
S	60 (67)	48 (53)	59 (67)	60 (68)	55 (62)
R	28 (31)	40 (46)	29 (33)	28 (32)	33 (38)

[a] S, sensitive; R, resistant.
[b] A total of 88 isolates were tested for each method.

TABLE 3 Interpretation of discrepant results between the MGIT 960 and Ion Torrent next-generation sequencing methods

Isolate no.	Ion Torrent	MGIT 960	Bactec 460	Wayne test[a]	Sanger
1	T −10 C	S[b]	R[c]	+	T −10 C
2	D12G	S	S	−	D12G
3	L35R	S	R	+	L35R
4	A102V	S	R	−	A102V
5	R154G	S	R	−	R154G
6	R154G/R (mixed)	S	R	+	WT

[a] +, Susceptible; −, resistant.
[b] S = sensitive.
[c] R = resistance.

Bactec 460 results (30 resistant, 44 susceptible, and 14 discrepant; $n = 88$), 83% of the Wayne enzymatic test results (23 resistant, 50 susceptible, and 15 discrepant; $n = 88$), and 91% of *pncA* gene Sanger sequencing results (26 resistant, 54 susceptible, and 8 discrepant; $n = 88$). One isolate exhibited resistance by the two phenotypic (MGIT 960 and Bactec 460) assays used in this study; however, no mutation was detected by either Sanger or Ion Torrent sequencing.

Ion Torrent sequencing detected mutations in 6 isolates that were not initially detected using Sanger sequencing (Table 3). This was probably as a result of the shorter length of the genetic region targeted by Sanger sequencing. One of these isolates exhibited double mixed mutations (L159P/L and T135P/T), three isolates exhibited mixed mutations (D12G/D, R154G/R, S59P/S), and two isolates had single mutations (S164P and C14R) on the *pncA* gene. The Bactec MGIT 960 PZA kit results showed 82% concordance with the Bactec 460 results (25 resistant, 43 susceptible, and 15 discrepant; $n = 83$), 84% concordance with the Wayne enzymatic test results, (20 resistant, 51 susceptible, and 14 discrepant; $n = 85$), and 85% concordance with the Sanger *pncA* sequencing results, (20 resistant, 52 susceptible, and 13 discrepant; $n = 85$).

DISCUSSION

Drug susceptibility testing for *M. tuberculosis* to pyrazinamide presently relies on phenotypic liquid culture methods that do not possess the sensitivity and specificity of whole-gene/genome next-generation sequencing. In this study, we show that NGS of the *pncA* gene provides additional diagnostic value and could be usefully considered in routine testing strategies for the management of MDR-TB. For example, previous studies have shown that common mutations in the *pncA* gene are located in three regions, 3 to 17, 61 to 85, and 132 to 142 (2, 8, 15). These three regions are important in formation of the active site of the PZase enzyme (1) and would manifest in the Wayne enzymatic assay. However, other regions might be involved. Of the 88 isolates sequenced using Ion Torrent, 55 isolates were wild type, and 33 isolates contained *pncA* gene mutations. The *pncA* mutations in these 33 isolates were shown to represent nucleotide substitutions (missense mutations), insertions, or deletions causing amino acid substitutions or frame shifts leading to nonsense polypeptides. The mutations were dispersed throughout the *pncA* gene. Also, recently, two new genes (*RpsA* and *panD*) that might be implicated in PZA resistance have been identified (16–18). The *RpsA* gene codes for a vital ribosomal protein involved in trans-translation (16–18). Trans-translation is involved in degradation of potentially toxic

protein products formed in stressed bacteria and is required for persistence and survival. The *panD* gene encodes an aspartate decarboxylase involved in synthesis of β-alanine, a precursor of pantothenate and coenzyme A (18).

Three isolates carried mutations in the *pncA* gene promoter region (T −10 C and T −12 C), and double mutations were noticed in three isolates. Additionally, there were seven heteroresistant isolates with resistant and susceptible amino acid changes. Some isolates had D8N, K96STOP, and H71Y mutations. Amino acids Asp 8 and Lys 96 are part of the catalytic triad Cys138-Asp8-Lys96, and are important for catalytic activation of *pncA* activity. Amino acid His 71 is involved in metal ion binding (19). This suggests that changes in these amino acids can result in PZA resistance in *M. tuberculosis*. Two isolates in this study exhibited the C14R mutation, which is a high-confidence mutation (15, 20). In addition to the C14R, mutation, S164P is also listed by Miotto et al. (20) as a mutation carrying a very high confidence (level A), serving as a prominent determinant of phenotypic PZA resistance. The confidence level of L151S as a resistance determinant, also detected in our study, is uncertain. We suggest that single-gene NGS might be adequate to detect these high-confidence mutations, and that whole-genome sequencing is not necessarily required.

The detailed profile of mutations in the *pncA* gene revealed by NGS in this study emphasizes the complexity of PZA resistance testing, which is apparent in the discrepant findings between NGS results and phenotypic tests. Of the 88 isolates used in this study, six isolates were sensitive by the MGIT 960 but had *pncA* gene mutations by Ion Torrent sequencing. One isolate was resistant by MGIT 960 testing, but was designated as wild type by Ion Torrent sequencing. This may, however, also be due to the use of a higher inoculum, which increases the pH of the PZA-containing media and neutralizes drug activity. These results agree with studies suggesting that MGIT 960 has shown incidents of false-positive PZA resistance (21–23). In our study, the sensitivity and specificity of MGIT 960 for detecting PZA resistance compared with Ion Torrent NGS as the gold standard were 82% and 96%, respectively. A discrepancy between the Bactec 460 (14/88 or 16%) system and Ion Torrent compared to that of the MGIT 960 (8/88 or 9%) was observed but was not significant.

Furthermore, Ion Torrent detected mutations missed by Sanger sequencing, probably because it allows for a high depth of coverage of the nucleotide compared to Sanger sequencing, which does not detect minor variants with a frequency lower than 20%.

The Wayne assay is simple to perform, cost-effective, and results are available within 7 days (24). However, in this study, high discrepancy was observed between the Ion Torrent and Wayne enzymatic assays (15/88 or 17%). A possible explanation might be that some PZA resistant isolates also showed a positive PZase test. Several studies (25–28) confirmed that PZA resistant isolates are not always PZAase negative. Also, certain PZA-susceptible isolates might show the absence of the PZase enzyme.

The Sanger sequencing method exhibited a similar rate of discrepancy (8/88 or 9%) to that of the MGIT 960. One isolate exhibited resistance by the two phenotypic (MGIT 960 and Bactec 460) assays used in this study; however, no mutation was detected by either Sanger or Ion Torrent sequencing. This could be due to the presence of other genes besides *pncA* responsible for PZA resistance (28).

From the data presented here, it can be concluded that tests such as the Bactec 460, MGIT 960, and Wayne test or Sanger sequencing methodology are likely to confirm only between 82% and 90% of actual PZA resistance as detected by NGS. A significant proportion of PZA-resistant isolates is being missed by other methods. The routine use of NGS in the diagnostic laboratory should be considered.

Study limitations. Two important aspects that might impact interpretation of our study findings need to be pointed out. First, we have not determined the strain lineage of the PZA-resistant isolates and cannot declare whether certain mutations observed are associated in any way with strain type. Second, we did not determine the degree of similarity between the cultured isolates and the isolates that could not be cultured in order to make a statement on the generalizability of the study results.

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