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

Nontuthuko E. Maningi,[a,b] Luke T. Daum,[c] John D. Rodriguez,[c] Matsie Mphahlele,[b*] Remco P.H. Peters,[a,d] Gerald W. Fischer,[c] James P. Chambers,[e] P. Bernard Fourie[a]#

University of Pretoria, Pretoria, South Africa[a]; South African Medical Research Council, Pretoria, South Africa[b]; Longhorn Vaccines & Diagnostics, San Antonio, Texas, USA[c]; Anova Health Institute, Johannesburg, South Africa[d]; University of Texas at San Antonio, San Antonio, Texas, USA[e]

Running Head: Next-generation sequencing of PZA resistance in TB

#Address correspondence to P Bernard Fourie, bernard.fourie@up.ac.za

*Present address: Jhpiego South Africa, Pretoria, South Africa

ABSTRACT

Technical limitations of common tests used for detecting pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis* (MTB) isolates pose challenges for comprehensive and accurate descriptions of drug resistance in patients with multi-drug resistant tuberculosis (MDR-TB) . In this study, a 606 base pair fragment (comprising the *pnc*A coding region plus promoter) was sequenced using Ion Torrent next generation sequencing (NGS) for detecting associated PZA resistance mutations in 90 re-cultured, MDR-TB isolates from an archived series collected in 2001. These 90 isolates were previously Sanger sequenced, with 55 (62%) designated as carrying wild type *pnc*A gene and 33 (38%) showing mutations. Also earlier, PZA susceptibility of the

isolates was determined using the Bactec 460 TB system and the Wayne test. In this study, isolates were re-cultured and susceptibility testing performed in Bactec 960 MGIT. Concordance between NGS and MGIT results was 87% (n = 90), and with the Bactec 460, Wayne test, and *pnc*A gene Sanger sequencing, 82% (n = 88), 83% (n = 88), and 89% (n = 88), respectively. NGS confirmed the majority of *pnc*A 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, *pnc*A mutations were located in the 151-360 region, and warrants further exploration. In these isolates, with known resistance to rifampicin, NGS of *pnc*A 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.

INTRODUCTION

Pyrazinamide (PZA) is a cornerstone first-line anti-tuberculosis compound commonly also used in second-line therapeutic treatment of multi-drug resistant *Mycobacterium tuberculosis* (MTB) 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 MTB *pnc*A 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 inhibition of membrane transport (3). The contribution of PZA to the killing of MTB as part of a multidrug regimen for the treatment of tuberculosis (TB) is considerable, and its inclusion with rifampicin in anti-TB therapeutic

regimens resulted in significant shortening of treatment duration from 18 to 6 months (3). The drug is known to specifically target semi-dormant bacteria that are not killed by other anti-tuberculosis drugs (4, 5).

Mutations in the *pnc*A gene are associated with phenotypically pyrazinamide-resistant isolates of MTB (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 multi-drug resistant (MDR) cases of TB is rarely based on 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 susceptibility testing of MTB to pyrazinamide, and specifically the use of the Bactec 960 Mycobaterium 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 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 *pnc*A gene mutations. (5, 12). In addition, the Bactec 460 radiometric culture assay and the Wayne enzymatic assay that 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 assessing the potential impact of PZA resistance on treatment outcomes in patients if 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 reinvestigated a collection of MTB isolates previously studied and characterized for PZA resistance using the Bactec 460 radiometirc assay, the Wayne test, and Sanger sequencing (12). We also aimed to detect heteroresistance by NGS, i.e. the co-existence 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 5866 isolates were tested for susceptibility to rifampicin (RIF), isoniazid (INH), streptomycin (STM) and ethambutol (EMB). Of these, 179 were labelled as multi-drug resistant (9.9% of these designated resistant to all four test drugs). All specimens were collected from South African patients, across eight of the nine provinces in the country. The 179 MDR-TB isolates were stored in Greave's media uninterrupted at -80°C freezer for 12 years since collection. Of these isolates, 130 were available for further investigation and 88 were successfully re-cultured after removal from storage. Frozen cultures were thawed for 24 hours, and 200 µl of each original culture suspension inoculated into Bactec 960 MGIT liquid culture media tubes. Only one passage was performed between Bactec 460 and MGIT 960 testing from original frozen cultures. The H37Rv reference strain was included as a positive control in this study. Pyrazinamide susceptibility of 86 of the 88 re-cultured isolates was previously

determined using the Bactec 460 TB System, whilst all 88 isolates had previously been tested using Wayne test and Sanger sequencing (10). In this study, we re-investigated PZA susceptibility using the Bactec 960 MGIT PZA Test Kit as per manufacturer's instructions, and also applied NGS of the *pnc*A gene for detection of confirmatory mutations.

MGIT positive isolates were checked for contamination by streaking on blood agar plates and incubating for 24 hours at 37°C. Following subculture, 0.5 ml MGIT medium containing MTB was transferred to 1.5 mL PrimeStore Molecular Transport Medium (MTM; Longhorn Vaccines & Diagnostics, San Antonio, Texas, USA), and shipped at ambient temperature to Longhorn Vaccines & Diagnostics, San Antonio, Texas (USA) for whole gene sequencing analysis using the Ion Torrent Personal Genome Machine (Life Technologies, Foster City, California, USA). PrimeStore MTM preserves sample nucleic acid integrity at ambient temperature for subsequent molecular analysis (13).

Phenotypic drug susceptibility testing. Pyrazinamide susceptibility testing by the Bactec MGIT 960 system using the Becton Dickenson commercial PZA kit was performed per manufacturer's instructions. Isolates of *M. tuberculosis* in MGITwere 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 upon growth unit values greater than or equal to 100 growth units (GU)≥100) or less than 100 GU, respectively.

Determination of genotypic resistance patterns. *DNA Extraction***.** Mycobacterial DNA was extracted from all 90 isolates using PrimeXtract (Longhorn Vaccines & Diagnostics, San

Antonio, Texas, USA). Briefly, 200 µl 100 % (v/v) ethanol, 200 µl lysis buffer, and 200 µl *Mycobacterium tuberculosis* inocula were transferred to a 1.5 ml micro-centrifuge tube. After thorough mixing and subsequent centrifugation, the entire supernatant was applied to a microextraction column, centrifuged for 1 minute at 13, 000 rpm, and the flow-through material was discarded. Wash buffer (200 µl) was applied to the extraction column, centrifuged for 1 min at 13,000 rpm, followed by further addition of wash buffer (200 µl) to the extraction column, with subsequent centrifuging in similar fashion as described above discarding the flow-through material. Total MTB DNA was eluted by 1 minute centrifugation at maximum speed using 50 ul of pre-heated (~75C) elution solution. Total MTB DNA was stored at -20℃ until used. *Amplification and sequencing of the amplified pncA gene*. The *pnc*A forward/reverse primers included *pnc*AF1, (5'-CGGATTTGTCGCTCACTAC-3') and *pnc*AR1, (5'-GCCGGAGACGATATCCAGAT -3'), comprising the full gene and also the *pnc*A promoter region. The expected size of the *pnc*A amplicon was 960 bases (14). PCR reactions were performed in a total volume of 50 µl, and the PCR reaction mixture consisted of 5 µl 10X buffer plus $MgCl_2$ (Longhorn Vaccines & Diagnostics, San Antonio, Texas, USA), 2 µl 20 µM forward and reverse *pnc*A primers (Integrated DNA Technologies, Coralville, Iowa, USA), 0.5µl Platinum Taq enzyme (Life Technologies, Grand Island, New York, USA), 35.5 µl nuclease free water (Integrated DNA Technologies, Coralville, Iowa, USA), and 5 µl extracted DNA. Amplification was performed on a ABI 9700 thermocycler (Life Technologies, Foster City, California, USA) under the following conditions: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 15 sec, and extension at 72 °C for 1 min with a final extension cycle at 72 °C for 5 min. PCR products were analysed by 1 % (w/v) agarose gel electrophoresis (Phoenix Research Products, Candler, North Carolina, USA),

and viewed using an Ultraviolet Transiluminator (Spectroline, Westberry, New York, USA).

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 *pnc*A sequencing. Library preparation was carried out using the Ion Xpress™ plus Fragment Library Kit (Cat# 4471269) for fragmentation and adaptor ligation with approximately 10-100 ng DNA amplicon. DNA amplicon was sheared using the IonXpress Shearing II kit modified as follows: 21.5 µl PCR template, 2.5 µl 10X shear buffer, and 1.0 µl shearing enzymes. Adapter ligation, nick repair, and amplification (8 cycles) were all performed per manufacturer's protocol. The prepared library was bar-coded using Ion Xpress Barcode Adaptors 1-96 (Cat# 4474517). The amount of library nucleic acid required for template preparation was made equal using Ion Library Equalizer Kit (Cat# 4482298) per manufacturer's instructions. Emulsion PCR and recovery steps were carried out using the Ion PGM Template OT2 400 kit (Cat# 4479878) according to 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 was performed using SeqMan NGen (V4) and LaserGene (V10) Core Suite (DNAStar, Madison, Wisconsim, USA).

RESULTS

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

Table 1: Bactec MGIT 960 PZA susceptibility test outcomes compared with Ion Torrent *pnc*A whole gene sequencing results

MGIT 960 Results*	Ion Torrent sequencing results* and description of mutations found	Number of strains
S	WT	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

* WT = wild type, S = susceptible, R = resistant

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 *pnc*A gene promoter by Ion Torrent NGS (Table 1). Ten substitutions were found in twelve isolates (C14R,

S164P, L35R, L515S, H71Y, D12G, D154G, A102V and D8Y, A79V). Four insertions/deletions were detected in five isolates in this study (Ins Tpos360, Ins Gpos35, Ins Gpos315 and Del Tpos515). Only one stop codon was found in one isolate (K96STOP). In this study 12 isolates had mutations within the 3-17 region, 3 isolates with mutations within the 61-85 region, and only 1 isolate with a mutation within the 132-142 region. The remaining isolates had mutations in the 151-360 region.

Historical PZA resistance results from other tests. For the 88 isolates included in this study, 46 (52%) were PZA susceptible by Bactec 460, 40 (46%) resistant, and 2 (2%) were borderline. By the Wayne enzymatic assay, 59 (67%) isolates were susceptible and 29 (33%) resistant, and by Sanger sequencing 60 (68%) susceptible and 28 (32%) 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 the Ion Torrent as the gold standard. Of the 8 discordant results, six isolates were susceptible by MGIT 960, but mutations were detected by 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 confirmation of 86% of Bactec 460 results (30 resistant, 44 susceptible, and 12 discrepant; n = 86), 83% of the Wayne enzymatic test results (23 resistant, 50 susceptible, and 18 discrepant; n = 88), and 91% of *pnc*A gene Sanger sequencing results (26 resistant, 54 susceptible, and 8 discrepant; n = 88). One isolate exhibited resistance by both phenotypic (MGIT 960 and Bactec 460) assays used in this study; however, no mutation was detected by either Sanger or Ion Torrent sequencing.

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

	MGIT 960 (N = 88)	Bactec 460 (N = 88)	Wayne Test (N = 88)	Sanger (N = 88)	Ion Torrent (N = 88)
S	60 (67%)	46 (53%)	59 (67%)	60 (68%)	55 (62%)
R	28 (31%)	40 (46%)	29 (33%)	28 (32%)	33 (38%)

S = sensitive; R = resistant

Ion Torrent detected mutations in 6 isolates that were not initially detected using Sanger sequencing (Table 3), 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 *pnc*A gene. The Bactec MGIT 960 PZA kit results showed 82% concordance with 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 Sanger *pnc*A sequencing results, (20 resistant, 52 susceptible, and 13 discrepant; n = 85).

Table 3: Interpretation of discrepant results between MGIT 960 and Ion Torrent Next Generation Sequencing methods

Isolate number	Ion Torrent	MGIT 960	Bactec 460	Wayne test	Sanger
1	T -10 C	S	R	+	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

S = sensitive, R = resistance, + = susceptible, - = resistant

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 *pnc*A gene provides additional diagnostic value and could be usefully considered in routine testing strategies for management of MDR-TB. For example, previous studies have shown that common mutations in the *pnc*A gene are located in three regions, 3–17, 61–85 and 132–142 (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 *pnc*A gene mutations. The *pnc*A 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 *pnc*A gene. Also, recently, two new genes (*Rps*A and *pan*D) that might be implicated in PZA resistance have been identified (16-18). The *Rps*A 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 *pan*D gene encodes an aspartate decarboxylase involved in synthesis of β-alanine, a precursor of pantothenate and co-enzyme A (18).

Three isolates carried mutations in the *pnc*A 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 both 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 *pnc*A 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 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 *pnc*A 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 *pnc*A gene mutations by Ion Torrent sequencing. One isolate was resistant by MGIT 960 testing, but 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, neutralising drug activity. These results agree with studies suggesting that MGIT 960 has shown incidents of false positive PZA resistance (21-23). In our study, sensitivity and specificity of MGIT 960 for detecting PZA resistance compared with Ion Torrent NGS as the 'gold standard' was 82% and 96%, respectively. A discrepancy between the Bactec 460 (12/88 or 14%) system, and Ion Torrent compared to that of the MGIT 960 (8/88 or 9%) was observed but these were not significant.

Furthermore, Ion Torrent detected mutations missed by Sanger sequencing, probably because it allows for a high depth of coverage of the nucleotide as compared to Sanger sequencing that 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 seven days (24). However in this study high discrepancy was observed between the Ion torrent and Wayne enzymatic assay (18/88 or 21%). 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 absence of the PZase enzyme.

The Sanger sequencing method exhibited a similar rate of discrepancy (8/88 or 9%) as the MGIT 960. One isolate exhibited resistance by both 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 *pnc*A responsible for PZA resistance (28).

From 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. Firstly, we have not determined strain lineage of the PZA resistant isolates and cannot declare whether certain mutations observed are associated in any way with

strain type. Secondly, 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.

ACKNOWLEDGEMENTS

NEM received PhD study bursaries from the University of Pretoria, the South African Medical Research Council, and the National Research Foundation of South Africa. She was also awarded a Global Infectious Diseases Research Training Fogarty Fellowship in 2013 which enabled access to next-generation sequencing laboratory facilities and training with LTD at Longhorn Vaccines and Diagnostics, San Antonio, Texas and JPC, The University of Texas at San Antonio.

REFERENCES

1. **Konno K, Feldmann FM, McDermott W.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis. **95**:461-469.
2. **Scorpio A, Zhang Y.** 1996. Mutations in *pnc*A, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med **2**:662-667.
3. **Zhang Y, Mitchison D.** 2003. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis **7**:6-21.
4. **Heifets L, Lindholm-Levy P.** 1992. Pyrazinamide sterilizing activity in vitro against semi-dormant *Mycobacterium tuberculosis* bacterial populations. Am Rev Respir Dis **145**:1223-1225.

5. **Louw GE, Warren RM, Donald PR, Murray MB, Bosman M, Van Helden PD, Young DB, Victor TC.** 2006. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. **Int J Tuberc Lung Dis** 10:802-807.
6. **Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C.** 1997. Mutation in pncA is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tubercle Lung Dis **78**:117-122.
7. **Portugal, I, Barreiro L, Moniz-Pereira J, Brum L.** 2004. pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates in Portugal. Antimicrob Agents Chemother **48**:2736-2738.
8. **Morlock GP, Crawford JT, Butler WR, Brim SE, Sikes D, Mazurek GH, Woodley CL, Cooksey RC.** 2000. Phenotypic characterization of pncA mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **44**:2291-2295.
9. **Salfinger M, Heifets LB.** 1988. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method. Antimicrob Agents Chemother **32**:1002-1004.
10. **Parrish NM, Carroll KC.** 2011. Role of the clinical mycobacteriology laboratory in diagnosis and management of tuberculosis in low-prevalence settings. J Clin Microbiol **49**:772-776.
11. **Global Laboratory Initiative.** 2014. Mycobacteriology Laboratory Manual, First Edition April 2014. Stop TB Partnership, Geneva, Switzerland

(http://www.stoptb.org/wg/gli/assets/documents/gli_mycobacteriology_lab_manual_web.pdf - accessed 2015-03-01).

12. **Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsaker T, Muthivhi T, Weyer K, Fourie PB, Grewal HM.** 2008. Pyrazinamide resistance among South African multidrug-resistant *Mycobacterium tuberculosis* isolates. J Clin Microbiol **46**:3459-3464.

13. **Daum LT, Worthy S, Yim KC, Nogueras M, Schuman RF, Choi YW, Fischer GW.** 2011. A clinical specimen collection and transport medium for molecular diagnostic and genomic applications. Epidemiol Infect **139**:1764-1773.

14. **Daum LT, Rodriguez JD, Worthy SA, Ismail NA, Omar SV, Dreyer AW, Fourie PB, Hoosen AA, Chambers JP, Fischer GW.** 2012. Next-generation ion torrent sequencing of drug resistance mutations in *Mycobacterium tuberculosis* strains. J Clin Microbiol **50**:3831-3837.

15. **Zhang H, Bi LJ, Li CY, Sun ZG, Deng JY, Zhang XE.** 2009. Mutations found in the pncA gene of *Mycobacterium tuberculosis* in clinical pyrazinamide-resistant isolates from a local region of China. J Int Med Res **37**:1430-1435.

16. **Shi W, Zhang X, Jiang X, Yuan H, Lee JC, Barry CE III, Wang H, Zhang W, Zhang Y.** 2011. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. Science **333**:1630-1632.

17. **Simons SO, A. Mulder, Van Ingen J, Boeree MJ, Van Soolingen SD.** 2013. Role of rpsA gene sequencing in diagnosis of pyrazinamide resistance. J Clin Microbiol **51**:382.

18. **Zhang Y, Shi W, Zhang W, Mitchison DA.** 2013. Mechanisms of pyrazinamide action and resistance. Microbiol Spectr **2**:1-12.

19. **Zhang H, Deng JY, Bi LJ, Zhou YF, Zhang ZP, Zhang CG, Zhang Y, Zhang XE.** 2008. Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase. FEBS J **275**:753-762.

20. **Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, Bakonyte D, Stakenas P, Pimkina E, Augustynowicz-Kopec´ E, Degano M, Ambrosi A, Hoffner S, Mansjö M, Werngren J, Rüsch-Gerdes S, Niemann S, Cirillo DM.** 2014. *Mycobacterium tuberculosis* pyrazinamide resistance determinants: a multicenter study. mBio **5**:e01819-14. doi:10.1128/mBio.01819-14.

21. **Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F.** 2010. Potential for erroneous results indicating resistance when using the Bactec MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J Clin Microbiol **48**:300-301.

22. **Piersimoni C, Mustazzolu C, Giannoni F, Bornigia S, Gherardi G, Fattorini L.** 2013. Prevention of false resistance results obtained in testing the susceptibility of *Mycobacterium tuberculosis* to pyrazinamide with the Bactec MGIT 960 system using a reduced inoculum. J Clin Microbiol **51**:291-294.

23. **Shenai S, Rodrigues C, Sadani M, Sukhadia N, Mehta A.** 2009. Comparison of phenotypic and genotypic methods for pyrazinamide susceptibility testing. Indian J Tuberc **56**:82-90.

24. **Wayne LG.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis **109**:147-151.

25. **Butler WR, Kilburn JO.** 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother **24**:600-601.

26. **Cui Z, Wang J, Lu J, Huang X, Zheng R, Hu Z.** 2013. Evaluation of methods for testing the susceptibility of clinical *Mycobacterium tuberculosis* isolates to pyrazinamide. J Clin Microbiol **51**:1374-1380.

27. **Sharma B, Pal N, Malhotra B, Vyas L, Rishi S.** 2010. Comparison of MGIT 960 and pyrazinamidase activity assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. Indian J Med Res **132**:72-76.

28. **Singh P, Wesley C, Jadaun GP, Malonia SK, Das R, Upadhyay P, Faujdar J, Sharma P, Gupta P, Mishra AK, Singh K, Chauhan DS, Sharma VD, Gupta UD, Venkatesan K, Katoch VM.** 2007. Comparative evaluation of Lowenstein-Jensen proportion method, BacT/ALERT 3D system, and enzymatic pyrazinamidase assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol **45**:76-80.