**Issues in *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing: Pyrazinamide**

**HIGHLIGHTS**

- The primary mechanism of PZA resistance is due to mutations in the *pncA* gene and promoter region.
- PZA growth-based DST using automated broth systems have issues with false resistance and reproducibility.
- Some molecular methods may not detect subpopulations of PZA resistant isolates, but WGS could provide this capability.
- Growth-based DST and *pncA* gene and promoter region sequencing together may provide the most reliable prediction of PZA susceptibility or resistance.

**BACKGROUND**

Pyrazinamide (PZA) is a critical component of first-line drug combination therapy for *Mycobacterium tuberculosis* complex (MTBC) including both susceptible and multidrug-resistant tuberculosis (MDR TB). Inclusion of PZA has shortened the previous 9–12 month chemotherapy regimen to six months.¹⁻⁴ PZA has a sterilizing effect due to its significant activity against non-replicating “persister” organisms or semi-dormant, slowly replicating bacilli at acid pH conditions (pH 5.5), killing bacilli that are not eliminated by other TB drugs, such as those found in acidic regions of acute inflammation.⁵⁻¹⁰

Pyrazinamide is a pro-drug which requires conversion to its active form of pyrazinoic acid (POA) by MTBC. Pyrazinamide enters the mycobacterial cell by passive diffusion and is subsequently transformed in the cytoplasm by the protein PncA, a non-essential intracellular nicotinamidase that has pyrazinamidase (PZase) activity, encoded by the *pncA* gene. POA accumulates in the cytoplasm and is actively expelled by a putative efflux pump. Outside of the bacilli, POA is protonated and re-enters the organism where the release of the protons occurs, resulting in an increasingly acidic cytoplasm and the accumulation of POA. This disrupts membrane permeability and transport, resulting in cellular damage.¹⁰⁻¹² While this mechanism of action has been the prevailing theory, others have proposed that POA may not be responsible for acidification of the cytoplasm yet may inhibit target(s) only essential to the bacteria under stress conditions (e.g., hypoxia).¹³⁻¹⁶ More recently, Gopal et al. found that POA bound to aspartate decarboxylase, PanD, in the bacterial cell, triggering its degradation and blocking biosynthesis of the essential Coenzyme A.¹⁷ [See Areas of Ongoing Research]

The primary mechanism of PZA resistance is due to mutations in the *pncA* gene and promoter region, identified by several studies in 70-97% of phenotypically PZA resistant isolates.¹³,¹⁸,¹⁹ Mutations in *pncA* and the promoter region affect the catalytic sites of the PZase enzyme and Fe2+ ion

The World Health Organization (WHO) has published a “Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance” (2021). This is the first document to provide a common, standardized reference for the interpretation of resistance to first and second-line drugs. The catalogue includes 17,000 mutations, their frequency and association with resistance. The mutations are further classified into two tiers. Tier 1 comprises genes considered most likely to contain the resistance mutations. Tier 2 comprises genes that are reasonably likely to contain resistance mutations, with the additional, literature-defined promoter sequences. The WHO document is an important resource with detailed information on numerous mutations associated with drug resistance and may include mutations not specifically addressed in this white paper. We recommend readers to review both documents if they are interested in molecular detection of drug resistance.
binding site resulting in reduction or loss of PZase activity.\textsuperscript{20–23} While the loss of PZase activity does not impact the fitness or viability of the organism, it does prevent conversion of PZA to POA.\textsuperscript{24–27}

Mutations in the \textit{pncA} gene are diverse and widely distributed throughout the gene and a variety of mutations have been correlated with high-level resistance. However, no particular “hot-spot” region has been identified and some mutations are not associated with loss of PZase function while others have been identified as “lineage-specific.”\textsuperscript{13,18,19,23,28–37} The non-essential nature of \textit{pncA} has likely allowed for the development of mutations across the entire gene and promoter region without affecting bacterial fitness.\textsuperscript{38} Whitfield et al. analyzed \textit{pncA} mutations that were not found to confer PZA resistance at the 100 \(\mu\)g/mL critical concentration in the MGIT 960 system (Becton-Dickinson) and observed that most of the \textit{pncA} polymorphisms associated with susceptible isolates identified in the study had a PZA minimum inhibitory concentration (MIC) between 50 and 100 \(\mu\)g/mL, just below the critical concentration.\textsuperscript{28} A systematic review summarized published mutations identified in \textit{pncA} and/or the \textit{pncA} promoter region associated with PZA resistance, estimating a global sensitivity and specificity of 83% and 90%, respectively.\textsuperscript{19,37} In a population-based retrospective multi-country surveillance project, levels of PZA resistance among TB cases, as assessed by sequencing of \textit{pncA}, varied from 3.0–42.1% depending on the setting.\textsuperscript{39} The recently published World Health Organization (WHO) “Catalogue of mutations in \textit{Mycobacterium tuberculosis} complex and their association with drug resistance” determined a sensitivity and specificity of 72.3% and 98.8%, respectively for the \textit{pncA} gene.\textsuperscript{37}

Not all \textit{pncA} mutations result in detectable PZA resistance by current growth-based drug susceptibility testing (DST)\textsuperscript{28} and some PZA resistant isolates may not have any \textit{pncA} or promoter region mutations (\textit{pncA}-WT) [See \textbf{Practical Laboratory Issues}]. Resistance may also be due to other mechanisms such as efflux of POA, which is dependent on level of PZase activity, intracellular PZase concentrations and POA efflux pump efficiency; altered PZA uptake; impaired POA binding to drug targets and \textit{pncA} gene expression.\textsuperscript{11,40–42} Other potential gene targets have been identified such as \textit{panD}, and \textit{clpC1}. To date, few evaluations of these targets in \textit{pncA}-WT, phenotypically PZA resistant isolates have determined that while these gene targets may have a role in MTBC PZA resistance, it is not fully understood and may only account for resistance in a small proportion of isolates.\textsuperscript{27,29,43–48} [See \textbf{Areas of Ongoing Research}]

Pyrazinamide monoresistance is often an indicator of \textit{Mycobacterium bovis} or \textit{Mycobacterium bovis} BCG, which are naturally resistant to PZA and contain a single mutation at nucleotide position 169 in \textit{pncA}, resulting in the His57Asp mutation.\textsuperscript{27,41,49}

\section*{PRACTICAL LABORATORY ISSUES}

\subsection*{Growth-Based Phenotypic PZA DST and Test Methods}

Current FDA cleared growth-based DST methods for PZA in the US include two commercial automated broth systems: BACTEC\textsuperscript{TM} MGIT\textsuperscript{TM} 960 (MGIT) with the BD BACTEC\textsuperscript{TM} MGIT 960 PZA kit (MGIT PZA assay, Becton Dickinson) and VersaTREK\textsuperscript{TM} Automated Microbial Detection System (VersaTREK) with the VersaTREK\textsuperscript{TM} Myco PZA kit (VersaTREK PZA assay, TREK Diagnostic Systems, ThermoFisher Scientific\textsuperscript{TM}). Other research-use only (RUO) or laboratory developed test (LDT) methods do not generally include PZA primarily due to the need for acidic media.

Liquid broth systems are the recommended and most commonly used method for first-line DST, including PZA.\textsuperscript{28} Specifically, the most commonly utilized method for PZA growth-based DST is the MGIT PZA Assay performed on the MGIT. This assay is based on growth of the MTBC isolate in a drug containing tube compared with a drug free tube (growth control). The MGIT 960 instrument continuously monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared with the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. The MGIT PZA Assay utilizes an acidified pH (approximately 5.9) modified Middlebrook 7H9 broth with growth supplement (bovine albumin, dextrose and polyoxyethylene stearate) and a modified proportion method, with a critical concentration of 100 \(\mu\)g/mL of PZA. The VersaTREK PZA assay has not been as widely evaluated as the MGIT PZA Assay.\textsuperscript{55} The VersaTREK PZA assay utilizes a standardized suspension of MTBC to inoculate into drug-containing VersaTREK Myco bottles and a drug-free control bottle which are monitored for growth. Growth is detected by a change in gas pressure in the headspace of the bottle due to the consumption of oxygen by the mycobacterial isolate. The VersaTREK PZA Assay utilizes an acidified medium (Middlebrook 7H9-based, pH 5.9–6.0) with growth supplement (MYCO GS) and a critical concentration of 300 \(\mu\)g/mL of PZA.

Due to normal variation in set-up, the present critical concentration used for PZA (100 \(\mu\)g/mL; MGIT PZA assay) may result in discrepancies for isolates that have a PZA MIC close to the critical concentration. The determination of an appropriate clinical breakpoint or MIC must rely on using a distribution of MICs from wild type MTBC strains, as clinical outcome data for treatment with PZA alone are not obtainable. WHO recognizes the MGIT assay as the only liquid culture method for PZA susceptibility testing, “even though a high rate of false-positive resistance results have been reported in some laboratories.”\textsuperscript{55} The WHO
definition of the critical concentration for PZA (MGIT PZA Assay) categorizes up to 10% of wild type MTBC strains as drug-resistant.51

Despite being the recommended platforms, there are several issues with PZA growth-based DST using automated broth systems; in particular, false resistance and difficulties with reproducibility due to the poor buffering of test media, use of acidic media at pH that inhibits growth, and large inoculum that reduces the activity of PZA.1,43,52–55 [See Considerations for PZA DST]

Other non-FDA cleared methods have been developed and assessed for the detection of PZA resistance. These include the resazurin microtiter assay (REMA), colorimetric nitrate reductase assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (MTT reduction test).44,56–61 The REMA and MTT assays detect bacterial growth in a microtiter well format through redox reactions. Recently, another group applied the Microplate Alamar Blue Assay (MABA), with nicotinamide as a surrogate for PZA in media at neutral pH and compared results to those obtained from the MGIT PZA Assay (with reduced inoculum), and pncA and promoter region gene analysis. MABA was comparable to results obtained using the MGIT PZA Assay and pncA sequencing.52

The PZase test (or Wayne test), non-FDA-cleared, detects the production of PZase through successful deamination of PZA to POA and ammonia. The reaction is observed as the formation of a pink band in the subsurface agar that diffuses into the medium, indicating enzymatic hydrolysis of PZA to free POA. Without the sufficient (required) inoculum, the PZase test can be misinterpreted, resulting in false resistance. Furthermore, studies have identified isolates with PZase activity that remain PZA-resistant, indicating resistance due to a mechanism independent of PZase. Test sensitivity has been reported to vary between 79–96%,24,44,53,63,64 A study comparing PZase activity to the historic reference method BACTEC 460 (discontinued radiometric liquid culture-based assay) concluded that negative PZase results could be used to determine PZA resistance, but care should be taken in the interpretation of PZase-positive results.65–67 Of note, this study has not been repeated as compared to currently available growth-based methods (MGIT PZA Assay or TREK PZA Assay).

A simplified PZA test has also been developed using liquid culture media and detection of PZase activity in media using the optical density measurement of color change. The assay can be performed on positive–flagged MGIT tubes. In 114 isolates evaluated, the authors report a 95% concordance with the MGIT PZA Assay and pncA mutations.68 Further studies are needed to better establish the relationship between growth-based DST and PZase testing.

### Molecular-based Genotypic PZA DST and Test Methods

Molecular detection of DNA mutations can provide valuable information to predict drug resistance, particularly for PZA given the challenges with growth-based DST methods. Current molecular methods used to identify PZA resistance are primarily focused on detection of mutations in pncA and the promoter region, although mutations have been identified in other genes (e.g., panD, clpC1. See Areas of Ongoing Research) including targeted and whole genome sequencing (WGS) methods. A line probe assay has been developed for the detection of mutations within the genetic loci associated with resistance to pyrazinamide (pncA and promoter region) but is not available in the US and therefore not further described here.23,69,70 The benefits of molecular-based assays include performing the assays directly on specimens or isolates in a relatively rapid amount of time. There are two general types of sequencing-based assays: targeted sequencing such as Sanger sequencing and targeted next generation sequencing (tNGS) and non-targeted or WGS. The targeted sequencing methods mentioned vary in their methodology, but they can detect specific “targeted” changes in a gene or loci. tNGS can be performed on clinical specimens due to the PCR amplification included in the method and isolates whereas WWGS is most commonly performed on isolates. Depending on workflows, targeted sequencing methods can have a shorter turnaround time and be more cost-effective. (See Ongoing Research: Sequencing Technologies to Identify Resistance for more details).

Molecular detection approaches for the identification of PZA resistance should minimally include complete sequencing of the pncA gene and promoter region. Recent studies and international efforts have focused on identifying and assessing the clinical relevance of mutations in pncA and other genes.13,23,29,33–37 Limitations of some molecular methods include a decreased ability to detect subpopulations of resistant isolates (i.e., a heterogeneous population of MTBC) as compared to growth-based DST. However, whole genome sequencing (WGS) could potentially identify some of these populations. A systematic review and other studies have identified that PZA resistance is ubiquitous and increases in prevalence as risk of resistance to other drugs increases. Molecular assays that target pncA can detect resistance in MDR-TB isolates with high positive predictive values and rule-out PZA resistance in non-MDR isolates with high negative predictive values.30

With all molecular methods, particularly sequence-based methods used as a stand-alone test or to confirm other DST results, the region(s) tested, and the analysis pipeline must be able to differentiate mutations that are known to confer resistance to PZA and those that are not known to predict resistance.
Table 1. Growth-based and Molecular-based Drug Susceptibility Methods for PZA

<table>
<thead>
<tr>
<th>Growth-based Method</th>
<th>Determination of Resistance</th>
<th>Commercially Available Test Systems-US (Regulatory Status)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated Broth System</td>
<td>Growth in the presence of PZA at the critical concentration of 100 µg/mL (MGIT PZA) and/or 300 µg/mL (VersaTREK PZA)</td>
<td>BD BACTEC™ MGIT 960 PZA Kit (FDA cleared), VersaTREK™ Myco PZA Kit (FDA cleared)</td>
</tr>
<tr>
<td>Pyrazinamidase (PZase) Activity</td>
<td>Lack of PZase activity</td>
<td>RUO³</td>
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<table>
<thead>
<tr>
<th>Molecular-based Method</th>
<th>Determination of Resistance</th>
<th>Commercially Available Test Systems-US (Regulatory Status)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger sequencing</td>
<td>Detection of specific mutations within the genetic loci and promoter region associated with resistance to PZA (pncA)⁶</td>
<td>LDT only</td>
</tr>
<tr>
<td>Targeted next generation sequencing (tNGS)</td>
<td>Detection of specific mutations within the genetic loci and promoter region associated with resistance to PZA (pncA)⁶</td>
<td>Deeplex® Myc-TB (RUO), Ion AmpliSeq™ TB Panel (RUO)⁶</td>
</tr>
<tr>
<td>Whole genome sequencing (WGS)</td>
<td>Detection of mutations within the genetic loci and promoter region of pncA and analyzing the entire genome for other genetic predictors of drug resistance (pncA)³⁴</td>
<td>LDT only</td>
</tr>
</tbody>
</table>

a Critical concentrations included in the table are from CLSI M24 unless noted otherwise.³⁵
b. Assays are commercially available as RUO but can be validated for clinical testing.
c. Other gene targets, such as panD, may contribute to PZA resistance.
d. Mutations detected in other potential targets/regions (e.g. reported in literature to be associated with PZA resistance) could be identified using this method.

Considerations for PZA DST

Drug susceptibility testing for PZA can be challenging. Reproducibility of PZA molecular DST results are inferior compared to performance with other first-line drugs.³⁶,⁵⁵ External quality assessment programs and surveillance have identified that PZA testing is the most prone to error among the first-line drugs and that some laboratories do not perform PZA growth-based DST due to increased costs and technical difficulties associated with the assay.⁷¹,⁷² The MGIT PZA Assay has known issues with specificity (i.e., false resistance), and even when repeat testing is performed false resistance can still occur.³⁵,⁵³,⁷³,⁷⁴ Repeat growth-based DST or pncA sequencing may be useful for confirmation, though not all pncA mutations confer resistance and some isolates are resistant due to other mechanisms.³⁵ The acidic environment required for PZA activity may inhibit the growth of MTBC isolates. Therefore, the MIC of PZA is pH-dependent and has been shown to increase with increasing pH. Additionally, one study demonstrated that growth-based DST for PZA at a decreased temperature (28ºC instead of 37ºC) could also improve reproducibility, but the time to positivity was longer and therefore not optimal for clinical use.³⁶

The reproducibility of PZA growth-based DST results are particularly affected by the size of the test inoculum.³⁵ A large inoculum (10⁷ cells/mL to 10⁸ cells/mL) has been shown to raise the pH of the medium to seven, such that PZA has poor in vitro activity resulting in false resistance. Conversely, if the inoculum is too low, organisms might not grow well in the low pH medium and may appear to be falsely susceptible. This should be identified by poor growth in the control tube as well as the PZA tube, rendering the result invalid. The most commonly used inoculum for DST (containing 106 cells/mL) and recommended by CLSI only results in a small pH increase (less than 0.3 units).³⁵ Growth-based DST should be performed using fresh subcultures, as older culture material may contain metabolically inactive organisms, and appear falsely susceptible. The presence of bovine serum albumin in media has been found to raise the pH of acidic media and binds to POA, which may result in false resistance.⁹,⁷⁵,⁷⁶

Hoffner et al. identified significant differences with PZA DST between experienced laboratories in a proficiency testing survey of five laboratories and a reference laboratory in Sweden. The most common error observed was false resistance.⁷⁷ Nikolayevskyy et al. reviewed 2010-2014 external quality assessment data from the European Union and identified that PZA was consistently the most problematic drug, comprising 5% of all growth-based DST errors.⁷³ Similar issues with reported false resistance to PZA by the MGIT PZA Assay are evident in the US Centers for Disease Control and Prevention’s (CDC) Model Performance Evaluation Program (MPEP) with MTBC drug susceptibility testing data. Between 2012 and 2019, 75 isolates were analyzed for PZA susceptibility using the MGIT PZA Assay. While 92.4% of data from the MGIT PZA Assay corresponded with the expected results, only 22 isolates (29%) yielded expected results by all laboratories. Moreover, less than 85% congruence was observed for 10
isolates (13%), indicating false resistance as compared to the expected results. 78–93

**Approaches to Improving Reproducibility and Accuracy Using Growth-based Methods**

**PZA DST Inoculum**

As outlined above, variation in the starting inoculum can affect results. If a much larger inoculum or non-homogeneous inoculum (clumps) is used for testing, there is an increased potential for obtaining a false resistance result. In order to ensure a uniform inoculum it has been suggested that the inoculum preparation be “standardized” to approximately 10^6 CFU/mL. This can be achieved by allowing the test inoculum to settle after removal of culture material from the primary MGIT tube, removing the supernatant and then diluting to a 0.5 McFarland standard. Piersimoni et al. used a reduced inoculum MGIT PZA Assay protocol (of 0.25 mL instead of 0.5 mL) and compared it to the standard MGIT PZA Assay protocol with an increased concentration of PZA (200 µg/mL instead of 100 µg/mL). While both protocols decreased the likelihood of false resistance, the reduced inoculum protocol (0.25 mL instead of 0.5 mL) provided the best separation between true- and false-resistant isolates.

Morlock et al. determined that false resistance is mitigated using reduced inoculum densities without reducing inoculum volume. However, any modifications resulted in an increase of time to test completion. In this study, the protocol was identical to the manufacturer’s recommendations, but the cell density of the inoculum was reduced. The most dilute inoculum tested (1:50 dilution of seed tube for growth control tube and 1:5 dilution of seed tube for PZA containing tube) was determined to provide fewer false-resistant results. Additionally, the authors determined that using a lower cell density inoculum did not increase the occurrence of false-susceptible results. However, they recommended if an isolate is found to be susceptible to PZA but resistant to other first-line drugs, it should be tested using the standard inoculum method. A summary of approaches to decrease PZA false resistance with the MGIT PZA Assay is also available. While limited data are available to determine if false resistance is also an issue with the TREK PZA Assay, it is likely that there are similar issues and similar approaches could be considered.

**Use of More Than One Test Concentration or Determination of MICs**

Due to normal variation in setup, the present PZA critical concentration (MGIT-100 µg/mL, TREK-300 µg/mL) may result in discrepancies for isolates that have a PZA MIC close to the critical concentration. Varying interpretations of results for the MGIT PZA Assay have been suggested, including an increased cutoff of 300 µg/mL, a range (100, 300 and 900 µg/mL corresponding to susceptible, intermediate and resistant) based on the historic reference method (BACTEC 460), or setting the critical concentration at 200 µg/mL based on the theoretical MIC at a pH of 6.0. Alternatively, Werngren et al. suggested interpreting MICs of less than 64 µg/mL as susceptible, less than 128 µg/mL as intermediate, and equal to or greater than 128 µg/mL as resistant based on a study of phenotypic resistance and pncA gene mutations in PZA resistant MTBC.

There is a need to further characterize the phenotype of isolates with specific pncA mutations and their correlation to MICs. Aono et al. recently reported the detection of sub-populations within strains that exhibited differing characteristics affecting initial PZA DST results. Also identified were three isolates where the pncA gene was deleted, with phenotypic resistance to PZA. The use of WGS is expanding knowledge which may result in better correlation of mutations within the pncA and the pncA promoter region association with resistance, and identification of other gene mutations and mechanisms of resistance. This critical information can be utilized to predict the efficacy of PZA for particular isolates, leading to personalized therapeutic regimens.

**Quality Assurance**

CLIA-certified laboratories must participate in a CLIA-approved proficiency testing (PT) program to satisfy regulatory requirements for DST performance. If a qualified program is not available a suitable alternative must be implemented, such as inter-laboratory comparison. Proficiency testing for PZA DST is not readily available and is not included as one of the WHO Network of Supranational Reference Laboratory’s yearly proficiency test panels. In the US, the College of American Pathologists offers PT for PZA DST and other anti-tuberculosis drugs; however, their program provides only two challenges per year. MPEP is an educational self-assessment tool offering five MTBC isolates per challenge which includes both drug resistant and drug susceptible isolates. It provides an opportunity to compare results to those obtained by other participants using the same methods. MPEP is not a formal, graded PT program, but could be used as an adjunct to the laboratory’s regulatory PT program.

**IMPACT ON CLINICAL OUTCOMES**

PZA is a critical component of first-line drug combination therapy for tuberculosis for both susceptible and MDR TB. PZA is also considered an important component of shorter, new drug regimens and can be used in combination with novel anti-tuberculosis
 drugs FDA approved for clinical treatment such as pretomanid in 2019 and bedaquiline in 2014.3,4

As PZA is typically used for a short amount of time (i.e., the first six to eight weeks of the recommended CDC treatment regimen for drug-susceptible TB), it is important that DST results are provided rapidly, accurately, and reliably. False resistance may result in unnecessary prolonged therapy. The lack of reliable PZA resistance data hampers efforts for determining priorities of new tuberculosis treatment regimens, determining the effectiveness of drug treatments using novel drugs and highlights the need for improved diagnostics for routine use in programmatic settings.106

**AREAS OF ONGOING RESEARCH**

**pncA**

Several studies and systematic reviews have provided ample evidence for the routine performance of pncA and promoter region sequencing or incorporation of sequencing along with phenotypic DST as part of the testing algorithm.13,15,17,25,33,34,63,74,106,107

Another approach is to rule-in PZA susceptibility by the detection of a wild type pncA gene.63,100 However, silent mutations preventing hybridization and detection present a concern for reporting false resistance.23 Additional research will result in an improved understanding of uncharacterized pncA mutations and of the contribution of mutations other than those found in pncA.

A functional approach has been taken by Li et al., who developed an assay that employs a rapid colorimetric detection of PCR-based in vitro-synthesized PZase based on the isolate’s pncA gene sequence. This assay is useful for identifying PZA resistance that is due to pncA mutations, but it cannot detect resistance due to other mechanisms.60,108

Havlíček et al. developed a rapid microarray-based assay in a closed cartridge system for detection of PZA resistance associated with pncA mutations.34 The assay was able to predict PZA susceptibility as inferred from a pncA wild type genotype with a specificity of 100% but is limited by the restricted target range or detection of unknown phylogenetic or other non-resistance conferring mutations as false positives. The primary advantage of this assay over other sequencing approaches is the closed cartridge system that offers reduced contamination risks and application as a rapid point-of-care test in resource-limited settings.34

In a small study, Tam et al. evaluated the performance of an in-house developed pncA sequencing assay for the detection of PZA resistance directly from MTBC NAAT-positive sputum specimens. A reported success rate of 88.6% was reported in all specimens (smear negative 86.2%, smear positive 100%), with an average turn-around time of four working days.109

**panD**

The panD gene, which encodes aspartate decarboxylase, has been identified as a target of POA by whole genome sequencing. panD has been shown to be critical for survival and persistence of MTBC in vivo and is required for Coenzyme A (CoA) synthesis, necessary for cellular metabolism. POA has been demonstrated to block CoA biosynthesis, interfering with important metabolic functions of the cell. M. canettii, intrinsically resistant to PZA, has been found to contain mutations in panD.15,17,45

**Other Potential Mechanisms of Resistance**

In addition to the targets mentioned above, other targets have been identified that are being evaluated for their association with PZA resistance including cypC1 and gpsL.15,17,110 Studies looking at the active component of PZA (POA) and how it acts in the cell have identified the POA efflux rate as a predictor of resistance. Assays that could rapidly detect the POA levels are in development.11,42

Other theories have postulated that POA may not be responsible for acidification of the cytoplasm yet may inhibit target(s) only essential to the bacteria under conditions of stress (e.g., hypoxia).13–16,37,111,112 One potential target was the ribosomal protein S1, RpsA (translated from the rpsA gene). RpsA is involved in trans-translation, a component of the degradation process of potentially toxic protein products formed in stressed bacteria, however later studies have since determined that PZA activity is independent of trans-translation and RpsA.13,15,17,29,40,45,110,113 As such, rpsA is no longer considered a candidate gene associated with phenotypic resistance.17,29,40,110,112,113 A recent review article outlines potential mechanisms of action of POA against M. tuberculosis and proposed/reported resistance mutations in M. tuberculosis.114

**Sequencing Technologies to Identify Resistance**

Whole genome sequencing (WGS) and targeted sequencing assays have added to the diverse landscape of MTBC mutation detection contributing to an understanding of PZA drug resistance.113 WGS is increasingly being successfully implemented in laboratories for routine clinical use for the prediction of TB drug susceptibilities. One large international study found the positive
predictive value (resistance) to PZA was 80.9%. As mutations responsible for PZA resistance are quite diverse and widespread along the \textit{pncA} locus, and other molecular mechanisms may be responsible for PZA resistance, the positive predictive value of WGS was not as high as that seen for other drugs. Conversely, the negative predictive value (susceptible) was excellent at 98.7%. The recently published “Catalogue of mutations in \textit{Mycobacterium tuberculosis} complex and their association with drug resistance” by WHO in 2021 determined a PPV to be 91.1% (overall performance). Other studies have similar findings. Recent studies examining the significance and clinical relevance of mutations associated with PZA—as well as other anti-tuberculosis drugs—have used a comprehensive approach encompassing microbiological, clinical and epidemiological data.

While the technical component of WGS has vastly improved in the past few years, the bioinformatics component is not fully standardized and can impact the predictive power of resistance prediction. There are efforts to create standardized approaches, but laboratories either must develop in-house tools and/or access currently available online tools. Recently, Iwamoto et al. examined online tools (i.e., TB Profiler, TGS-TB, PhyResSE and CASTB) and determined that the cause of reported low sensitivity for PZA resistance prediction was the tool interpretation pipeline and the pre-defined mutation catalogues, not the inability of software algorithms to detect genetic variants. The authors provide approaches how to improve the utility of these tools.

In addition to using WGS on isolates, efforts to sequence directly from sputum specimens for drug susceptibility prediction have achieved some success. In one study, WGS using targeted enrichment was able to provide sequences for 74% of specimens received within five days of specimen collection. The quality of the sequence data strongly correlated with the input level of TB DNA. Complete concordance was found between resistance mutations identified in paired MGIT and sputum specimens from nine participants when there was >85% single read coverage against the reference genome. Also, a significant number of minority single nucleotide variants in sputum were detected compared to the matched MGIT specimen sequence.

In addition to WGS, there are also tNGS approaches available including commercial methods such as the Ion AmpliSeq™ TB Panel. The assay includes \textit{pncA} gene analysis; a small study evaluating the method found reasonable concordance between genotypic prediction and phenotypic (PZA susceptibility estimated using PZase activit test). Deeplex\textsuperscript{®} Myc-TB, another commercial assay, involves the amplification and sequencing of 22 mycobacterial loci including 18 gene targets known to be involved in MTBC resistance to first and second line drugs. A recent study examining MDR TB utilized this assay, as well as WGS, with both methods identifying genotypic resistance that had not previously been detected using WHO-endorsed commercial assays.

WGS approaches combined with protein expression and functional genomics may provide additional gene targets involved in PZA resistance. For more information about WGS for molecular DST, refer to the WHO "Technical guide on next-generation sequencing technologies for the detection of mutations associated with drug resistance in \textit{Mycobacterium tuberculosis} complex."

**Critical Concentration and Breakpoints**

Research challenging the current critical concentration testing protocols and the development of new testing breakpoints is ongoing. Further determination of more accurate distribution of MICs in wild type MTBC and the relationship to clinical outcomes is needed.

**GUIDANCE**

Laboratories should consider the following:

- Use fresh cultures for preparation of test inoculum for growth-based DST.
- Ensure a standard inoculum preparation of approximately 10\textsuperscript{6} CFU/mL for DST.
  - Allow the test inoculum to settle (10-30 minutes) after removal of culture material from the primary MGIT tube, remove supernatant and then dilute to a 0.5 McFarland standard.
  - If preparing inoculum from solid media, a suspension of the isolate should be prepared in broth, vortexed thoroughly with glass beads (2-5 minutes) to break up clumps and allowed to settle (10-30 minutes). To avoid transferring clumps of MTBC, transfer the supernatant to a new sterile tube without disturbing the sediment (some laboratories perform this step twice with additional time for settling). Dilute the suspension to a 0.5 McFarland standard.
♦ Consider using a nephelometer or other measure of turbidity to standardize inoculum density. The presence of clumps in the suspension, or preparation of a suspension greater than 0.5 McFarland can increase the potential for false resistant results.

- Consider validating alternative inoculum preparations.\(^{35,53,55,94-97}\)

- If performing PZA DST from a MTBC positive MGIT, consider the following:\(^97\)
  - Use a day 1 culture (rather than day 2) OR
  - Use a day 3 culture diluted 1:5 (rather than day 4 or 5).
  - If resistant, consider repeat testing using
    - a day 3 culture.
    - a reduced inoculum.\(^{35,53,55,73,94,96-98}\)

- A combination of growth-based DST and \textit{pncA} and promoter region sequence-based testing may provide the most reliable and accurate prediction of PZA susceptibility or resistance:\(^{35,55}\)
  - Consider any isolates with non-synonymous \textit{pncA} gene and promoter region mutations as “PZA-resistant”, unless the mutation is a known lineage marker or not associated with resistance.\(^{35,36}\)
  - Repeat growth-based DST for any phenotypic PZA resistant isolates with synonymous \textit{pncA} mutations or \textit{pncA}-WT.\(^{74}\)
  - Consider WGS for isolates that are phenotypically resistant but \textit{pncA} and promoter region WT to identify other potential mutations associated with resistance (e.g. \textit{panD} mutations) or for some strains where intrinsic low-level resistance may be attributed to the genetic background.

- All monoresistant PZA isolates should be investigated and identified to the species level to determine if the isolate is \textit{M. bovis} or \textit{M. bovis} BCG (re-test using a day 3 culture).

- If a laboratory is unable to perform molecular testing for PZA, specimens or isolates can be referred to a jurisdictional public health laboratory for testing and/or those laboratories can refer to the following services:
  - CDC [Molecular Detection of Drug Resistance Service (MDDR)] provides rapid algorithm-based testing and other comprehensive testing services at no cost to all public health laboratories.
  - The [National Public Health Laboratory Drug Susceptibility Testing Reference Center for Mycobacterium tuberculosis] provides rapid algorithm-based testing at no cost to enrolled public health laboratories. Enrollment is restricted to public health laboratories performing TB DST on fewer than 50 isolates/year.
ABBREVIATIONS

CDC  US Centers for Diseases Control and Prevention
CFU  Colony forming units
DST  Drug susceptibility testing
CLIA  Clinical Laboratory Improvements Amendment
CLSI  Clinical and Laboratory Standards Institute
FDA  US Food and Drug Administration
LDT  Laboratory developed test
MDR TB  Multidrug-resistant tuberculosis
MGIT  Mycobacterium growth indicator tube
MIC  Minimum inhibitory concentration
MPEP  Model Performance Evaluation Program
MTBC  Mycobacterium tuberculosis complex
NAAT  Nucleic acid amplification test
POA  Pyrazinoic acid
PT  Proficiency testing
PZA  Pyrazinamide
PZase  Pyrazinamidase
RUO  Research use only
TB  Tuberculosis
tNGS  Targeted next generation sequencing
WGS  Whole genome sequencing
WT  Wildtype
WHO  World Health Organization

REFERENCES


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