# 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.[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.[24–27]

Mutations in the *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."[13,18,19,23,28–37] The non-essential nature of *pncA* has likely allowed for the development of mutations across the entire gene and promoter region without affecting bacterial fitness.[38] Whitfield et al. analyzed *pncA* mutations that were not found to confer PZA resistance at the 100 µg/mL critical concentration in the MGIT 960 system (Becton-Dickinson) and observed that most of the *pncA* polymorphisms associated with susceptible isolates identified in the study had a PZA minimum inhibitory concentration (MIC) between 50 and 100 µg/mL, just below the critical concentration.[28] A systematic review summarized published mutations identified in *pncA* and/or the *pncA* promoter region associated with PZA resistance, estimating a global sensitivity and specificity of 83% and 90%, respectively.[19,37] In a population-based retrospective multi-country surveillance project, levels of PZA resistance among TB cases, as assessed by sequencing of *pncA*, varied from 3.0-42.1% depending on the setting.[39] The recently published World Health Organization (WHO) "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance" determined a sensitivity and specificity of 72.3% and 98.8%, respectively for the *pncA* gene.[37]

Not all *pncA* mutations result in detectable PZA resistance by current growth-based drug susceptibility testing (DST)[28] and some PZA resistant isolates may not have any *pncA* or promoter region mutations (*pncA*-WT) [See 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 *pncA* gene expression.[11,40–42] Other potential gene targets have been identified such as *panD*, and *clpC1*. To date, few evaluations of these targets in *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.[27,29,43–48] [See Areas of Ongoing Research]

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

# PRACTICAL LABORATORY ISSUES

## 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™ MGIT™ 960 (MGIT) with the BD BACTEC™ MGIT 960 PZA kit (MGIT PZA assay, Becton Dickinson) and VersaTREK™ Automated Microbial Detection System (VersaTREK) with the VersaTREK™ Myco PZA kit (VersaTREK PZA assay, TREK Diagnostic Systems, ThermoFisher Scientific™). 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.[35] 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 µg/mL of PZA. The VersaTREK PZA assay has not been as widely evaluated as the MGIT PZA Assay.[50] 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 µg/mL of PZA.

Due to normal variation in set-up, the present critical concentration used for PZA (100 µg/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."[51] 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.[62]

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.[33,69,70] 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[a]

| Growth-based Method | Determination of Resistance | Commercially Available Test Systems-US (Regulatory Status) |
|---|---|---|
| Automated Broth System | Growth in the presence of PZA at the critical concentration of 100 µg/mL (MGIT PZA) and/or 300 µg/mL (VersaTREK PZA) | BD BACTEC™ MGIT 960 PZA Kit (FDA cleared), VersaTREK™ Myco PZA Kit (FDA cleared) |
| Pyrazinamidase (PZase) Activity | Lack of PZase activity | RUO[b] |
| **Molecular-based Method** | **Determination of Resistance** | **Commercially Available Test Systems-US (Regulatory Status)** |
| Sanger sequencing | Detection of specific mutations within the genetic loci and promoter region associated with resistance to PZA (*pncA*)[c] | LDT only |
| Targeted next generation sequencing (tNGS) | Detection of specific mutations within the genetic loci and promoter region associated with resistance to PZA (*pncA*)[b,c] | Deeplex® Myc-TB (RUO), Ion AmpliSeq™ TB Panel (RUO)[b] |
| Whole genome sequencing (WGS) | 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*)[b-d] | LDT only |

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 (28°C instead of 37°C) could also improve reproducibility, but the time to positivity was longer and therefore not optimal for clinical use.[16]

The reproducibility of PZA growth-based DST results are particularly affected by the size of the test inoculum.[55] A large inoculum ($10^7$ cells/mL to $10^8$ 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.[71] Similar issues with reported false resistance to PZA by the MGIT PZA Assay are evidenced 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.[35,53,73,77,94] 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.[35,95,96] 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.[94]

Morlock et al. determined that false resistance is mitigated using reduced inoculum densities without reducing inoculum volume.[97] 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.[97] A summary of approaches to decrease PZA false resistance with the MGIT PZA Assay is also available.[98] 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.[50]

### 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.[75,76,94] 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.[99] There is a need to further characterize the phenotype of isolates with specific *pncA* mutations and their correlation to MICs.[28,100] 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.[38] 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.[101–104] [See Areas of Ongoing Research]

## 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.[105]

# 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. [18,19,25,33,34,63,74,106,107] 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.[21] 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]

Havlicek 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*.[12,17,45]

## 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 *clpC1* 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 *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%.[101] The recently published "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance" by WHO in 2021 determined a PPV to be 91.1% (overall performance).[38] Other studies have similar findings.[102,103] 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.[23,29,31,101,103,115]

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.[116]

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.[117] 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 *pncA* gene analysis; a small study evaluating the method found reasonable concordance between genotypic prediction and phenotypic (PZA susceptibility estimated using PZase activit test).[118] Deeplex® 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.[119] 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.[120]

WGS approaches combined with protein expression and functional genomics may provide additional gene targets involved in PZA resistance.[12,121] 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 *Mycobacterium tuberculosis* complex."[122]

## 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.[99,123]

# 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 $10^6$ 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.[124,125]

        - 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 *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 *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 *pncA* mutations or *pncA*-WT.[74]
    - Consider WGS for isolates that are phenotypically resistant but *pncA* and promoter region WT to identify other potential mutations associated with resistance (e.g. *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 *M. bovis* or *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


1. World Health Organization. WHO consolidated guidelines on drug-resistant tuberculosis treatment. Geneva: World Health Organization; 2019. Report No.: WHO/CDS/TB/2019.7. Available from: https://www.who.int/tb/publications/2019/consolidated-guidelines-drug-resistant-TB-treatment/en/
2. World Health Organization. Guidelines for treatment of drug-susceptible tuberculosis and patient care (2017 update) Geneva: World Health Organization; 2017. Available from: http://www.who.int/tb/publications/2017/dstb_guidance_2017/en/
3. Diacon AH, Dawson R, von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, et al. Bactericidal Activity of Pyrazinamide and Clofazimine Alone and in Combinations with Pretomanid and Bedaquiline. Am J Respir Crit Care Med. 2015 Jan 26;191(8):943–53.
4. Olaru ID, Groote-Bidlingmaier F von, Heyckendorf J, Yew WW, Lange C, Chang KC. Novel drugs against tuberculosis: a clinician's perspective. Eur Respir J. 2015 Apr 1;45(4):1119–31.
5. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. Am Rev Respir Dis. 1992 May;145(5):1223–5.
6. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985 Sep;66(3):219–25.
7. Salfinger M, Heifets LB. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method. Antimicrob Agents Chemother. 1988 Jul;32(7):1002–4.
8. Salfinger M, Reller LB, Demchuk B, Johnson ZT. Rapid radiometric method for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. Res Microbiol. 1989 Jun;140(4–5):301–9.

9. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2003 Jan;7(1):6–21.

10. Zhang Y, Shi W, Zhang W, Mitchison D. Mechanisms of Pyrazinamide Action and Resistance. Microbiol Spectr. 2013;2(4):1–12.

11. Sheen P, Lozano K, Gilman RH, Valencia HJ, Loli S, Fuentes P, et al. *pncA* gene expression and prediction factors on pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuberc Edinb Scotl. 2013 Sep;93(5):515–22.

12. Zhang S, Chen J, Shi W, Liu W, Zhang W, Zhang Y. Mutations in panD encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*. Emerg Microbes Infect. 2013 Jun 12;2(6):e34.

13. Unissa AN, Hanna LE. Molecular mechanisms of action, resistance, detection to the first-line anti tuberculosis drugs: Rifampicin and pyrazinamide in the post whole genome sequencing era. Tuberc Edinb Scotl. 2017;105:96–107.

14. Peterson ND, Rosen BC, Dillon NA, Baughn AD. Uncoupling Environmental pH and Intrabacterial Acidification from Pyrazinamide Susceptibility in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2015 Dec;59(12):7320–6.

15. Anthony RM, den Hertog AL, van Soolingen D. "Happy the man, who, studying nature's laws, Thro" known effects can trace the secret cause.' Do we have enough pieces to solve the pyrazinamide puzzle? J Antimicrob Chemother. 2018 01;73(7):1750–4.

16. den Hertog AL, Menting S, Pfeltz R, Warns M, Siddiqi SH, Anthony RM. Pyrazinamide Is Active against *Mycobacterium tuberculosis* Cultures at Neutral pH and Low Temperature. Antimicrob Agents Chemother. 2016;60(8):4956–60.

17. Gopal P, Sarathy JP, Yee M, Ragunathan P, Shin J, Bhushan S, et al. Pyrazinamide triggers degradation of its target aspartate decarboxylase. Nat Commun 2020 Apr 3;11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7125159/

18. Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, et al. *Mycobacterium tuberculosis* Pyrazinamide Resistance Determinants: a Multicenter Study. mBio. 2014 Oct 31;5(5):e01819-14.

19. Ramirez-Busby SM, Valafar F. Systematic review of mutations in pyrazinamidase associated with pyrazinamide resistance in *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother. 2015 Sep;59(9):5267–77.

20. Petrella S, Gelus-Zietal N, Maudry A, Laurans C, Boudjelloul R, Sougakoff W. Crystal structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural and acquired resistance to pyrazinamide. PloS One. 2011;6(1):e15785.

21. Quiliano M, Gutierrez AH, Gilman RH, López C, Evangelista W, Sotelo J, et al. Structure-Activity relationship in mutated pyrazinamidases from *Mycobacterium tuberculosis*. Bioinformation. 2011;6(9):335–9.

22. Unissa AN, Selvakumar N, Hassan S. Insight to pyrazinamide resistance in *Mycobacterium tuberculosis* by molecular docking. Bioinformation. 2010;4(1):24–9.

23. Yadon AN, Maharaj K, Adamson JH, Lai Y-P, Sacchettini JC, Ioerger TR, et al. A comprehensive characterization of *pncA* polymorphisms that confer resistance to pyrazinamide. Nat Commun. 2017 19;8(1):588.

24. Cheng S-J, Thibert L, Sanchez T, Heifets L, Zhang Y. *pncA* Mutations as a Major Mechanism of Pyrazinamide Resistance in *Mycobacterium tuberculosis*: Spread of a Monoresistant Strain in Quebec, Canada. Antimicrob Agents Chemother. 2000 Mar 1;44(3):528–32.

25. Juréen P, Werngren J, Toro J-C, Hoffner S. Pyrazinamide Resistance and *pncA* Gene Mutations in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2008 May 1;52(5):1852–4.

26. Morlock GP, Crawford JT, Butler WR, Brim SE, Sikes D, Mazurek GH, et al. Phenotypic Characterization of *pncA* Mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2000 Sep;44(9):2291–5.

27. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med. 1996 Jun;2(6):662–7.

28. Whitfield MG, Warren RM, Streicher EM, Sampson SL, Sirgel FA, Helden PD van, et al. M*ycobacterium tuberculosis pncA* polymorphisms that do not confer pyrazinamide resistance at a breakpoint concentration of 100 µg/ml in MGIT. J Clin Microbiol. 2015 Aug 19;JCM.01001-15.

29. Ramirez-Busby SM, Rodwell TC, Fink L, Catanzaro D, Jackson RL, Pettigrove M, et al. A Multinational Analysis of Mutations and Heterogeneity in PZase, RpsA, and PanD Associated with Pyrazinamide Resistance in M/XDR *Mycobacterium tuberculosis*. Sci Rep. 2017 19;7(1):3790.

30. Whitfield MG, Soeters HM, Warren RM, York T, Sampson SL, Streicher EM, et al. A Global Perspective on Pyrazinamide Resistance: Systematic Review and Meta-Analysis. PloS One. 2015;10(7):e0133869.

31. Miotto P, Tessema B, Tagliani E, Chindelevitch L, Starks AM, Emerson C, et al. A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. Eur Respir J. 2017;50(6).

32. Ezewudo M, Borens A, Chiner-Oms Á, Miotto P, Chindelevitch L, Starks AM, et al. Integrating standardized whole genome sequence analysis with a global *Mycobacterium tuberculosis* antibiotic resistance knowledgebase. Sci Rep. 2018 18;8(1):15382.

33. Driesen M, Kondo Y, de Jong BC, Torrea G, Asnong S, Desmaretz C, et al. Evaluation of a novel line probe assay to detect resistance to pyrazinamide, a key drug used for tuberculosis treatment. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2018 Jan;24(1):60–4.

34. Havlicek J, Dachsel B, Slickers P, Andres S, Beckert P, Feuerriegel S, et al. Rapid microarray-based assay for detection of pyrazinamide resistant *Mycobacterium tuberculosis*. Diagn Microbiol Infect Dis. 2019 Jun;94(2):147–54.

35. Clinical Laboratory Standards Institute (CLSI). M24 Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic Actinomycetes 3rd ed. Clinical and Laboratory Standards Institute; 2018. Available from: https://clsi.org/standards/products/microbiology/documents/m24/

36. Warshauer DM, Salfinger M, Desmond E, Lin S-YG. *Mycobacterium tuberculosis* Complex. In: Manual of Clinical Microbiology, 12th Edition. Washington, DC: ASM Press; 2019. Available from: https://www.clinmicronow.org/doi/book/10.1128/9781683670438.MCM

37. World Health Organization. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. Geneva; 2021. Available from: https://www.who.int/publications/i/item/9789240028173

38. Aono A, Chikamatsu K, Yamada H, Kato T, Mitarai S. Association between *pncA* gene mutations, pyrazinamidase activity, and pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2014 Aug;58(8):4928–30.

39. Zignol M, Dean AS, Alikhanova N, Andres S, Cabibbe AM, Cirillo DM, et al. Population-based resistance of *Mycobacterium tuberculosis* isolates to pyrazinamide and fluoroquinolones: results from a multicountry surveillance project. Lancet Infect Dis. 2016 Oct;16(10):1185–92.

40. Shi W, Zhang X, Jiang X, Ruan H, Barry CE, Wang H, et al. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*: a potential mechanism for shortening the duration of tuberculosis chemotherapy. Science. 2011 Sep 16;333(6049):1630–2.

41. Somoskovi A, Dormandy J, Parsons LM, Kaswa M, Goh KS, Rastogi N, et al. Sequencing of the *pncA* gene in members of the *Mycobacterium tuberculosis* complex has important diagnostic applications: Identification of a species-specific *pncA* mutation in "*Mycobacterium canettii*" and the reliable and rapid predictor of pyrazinamide resistance. J Clin Microbiol. 2007 Feb;45(2):595–9.

42. Zimic M, Loli S, Gilman RH, Gutierrez A, Fuentes P, Cotrina M, et al. A new approach for pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*. Microb Drug Resist Larchmt N. 2012 Aug;18(4):372–5.

43. Bhuju S, Fonseca L de S, Marsico AG, de Oliveira Vieira GB, Sobral LF, Stehr M, et al. *Mycobacterium tuberculosis* isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the *pncA* gene. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2013 Oct;19:1–6.

44. Cui Z, Wang J, Lu J, Huang X, Zheng R, Hu Z. Evaluation of methods for testing the susceptibility of clinical *Mycobacterium tuberculosis* isolates to pyrazinamide. J Clin Microbiol. 2013 May;51(5):1374–80.

45. Feuerriegel S, Köser CU, Richter E, Niemann S. *Mycobacterium canettii* is intrinsically resistant to both pyrazinamide and pyrazinoic acid. J Antimicrob Chemother. 2013 Jun;68(6):1439–40.

46. Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. J Clin Microbiol. 2012 Nov;50(11):3726–8.

47. Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soolingen D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. J Clin Microbiol. 2013 Jan;51(1):382.

48. Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, et al. Role of *pncA* and *rpsA* Gene Sequencing in Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis* Isolates from Southern China. J Clin Microbiol. 2014 Jan;52(1):291–7.

49. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1997 Mar;41(3):540–3.

50. Espasa M, Salvadó M, Vicente E, Tudó G, Alcaide F, Coll P, et al. Evaluation of the VersaTREK System Compared to the Bactec MGIT 960 System for First-Line Drug Susceptibility Testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2012 Feb 1;50(2):488–91.

51. World Health Organization. Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis. Geneva: World Health Organization; 2018. Report No.: WHO/CDS/TB/2018.24. Available from: http://www.who.int/tb/publications/2018/WHO_technical_drug_susceptibility_testing/en/

52. Banu S, Rahman SMM, Khan MSR, Ferdous SS, Ahmed S, Gratz J, et al. Discordance across several methods for drug susceptibility testing of drug-resistant *Mycobacterium tuberculosis* isolates in a single laboratory. J Clin Microbiol. 2014 Jan;52(1):156–63.

53. Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F. Potential for Erroneous Results Indicating Resistance When Using the Bactec MGIT 960 System for Testing Susceptibility of *Mycobacterium tuberculosis* to Pyrazinamide. J Clin Microbiol. 2010 Jan;48(1):300–1.

54. Parsons LM, Somoskövi A, Urbanczik R, Salfinger M. Laboratory diagnostic aspects of drug resistant tuberculosis. Front Biosci J Virtual Libr. 2004 Sep 1;9:2086–105.

55. Woods GL, Lin S-YG, Brown-Elliott BA, Desmond EP. Susceptibility Test Methods: Mycobacteria, Nocardia, and Other Actinomycetes. In: Manual of Clinical Microbiology, 12th Edition. Washington, DC: ASM Press; 2019. Available from: https://www.clinmicronow.org/doi/book/10.1128/9781683670438.MCM

56. Abate G, Mshana RN, Miörner H. Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 1998 Dec;2(12):1011–6.

57. Mshana RN, Tadesse G, Abate G, Miörner H. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 1998 May;36(5):1214–9.

58. Rivoire N, Ravololonandriana P, Rasolonavalona T, Martin A, Portaels F, Ramarokoto H, et al. Evaluation of the resazurin assay for the detection of multidrug-resistant *Mycobacterium tuberculosis* in Madagascar. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2007 Jun;11(6):683–8.

59. Syre H, Øvreås K, Grewal HMS. Determination of the susceptibility of *Mycobacterium tuberculosis* to pyrazinamide in liquid and solid media assessed by a colorimetric nitrate reductase assay. J Antimicrob Chemother. 2010 Apr;65(4):704–12.

60. Zhou M, Geng X, Chen J, Wang X, Wang D, Deng J, et al. Rapid Colorimetric Testing for Pyrazinamide Susceptibility of *M. tuberculosis* by a PCR-Based In-Vitro Synthesized Pyrazinamidase Method. PLoS ONE. 2011 Nov 10;6(11):e27654.

61. Pina RZ, Caleffi-Ferracioli KR, Campanerut-Sá P a. Z, Ghiraldi-Lopez LD, Pavan FR, Siqueira VLD, et al. Pyrazinamide susceptibility testing in *Mycobacterium tuberculosis* using the fast resazurin microtiter assay plate. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016;20(11):1535–8.

62. Hu Y, Wu X, Luo J, Fu Y, Zhao L, Ma Y, et al. Detection of pyrazinamide resistance of *Mycobacterium tuberculosis* using nicotinamide as a surrogate. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2017 Nov;23(11):835–8.

63. Chang KC, Yew WW, Zhang Y. Pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*: a systematic review with meta-analyses. Antimicrob Agents Chemother. 2011 Oct;55(10):4499–505.

64. Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis. 1974

Jan;109(1):147–51.

65. Mestdagh M, Fonteyne PA, Realini L, Rossau R, Jannes G, Mijs W, et al. Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1999 Sep;43(9):2317–9.

66. Aono A, Hirano K, Hamasaki S, Abe C. Evaluation of BACTEC MGIT 960 PZA medium for susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide (PZA): compared with the results of pyrazinamidase assay and Kyokuto PZA test. Diagn Microbiol Infect Dis. 2002 Dec;44(4):347–52.

67. Davies AP, Billington OJ, McHugh TD, Mitchison DA, Gillespie SH. Comparison of Phenotypic and Genotypic Methods for Pyrazinamide Susceptibility Testing with *Mycobacterium tuberculosis*. J Clin Microbiol. 2000 Oct;38(10):3686–8.

68. Aono A, Chikamatsu K, Yamada H, Igarashi Y, Murase Y, Takaki A, et al. A simplified pyrazinamidase test for pyrazinamide drug susceptibility in *Mycobacterium tuberculosis*. J Microbiol Methods. 2018;154:52–4.

69. Matsumoto T, Ogata H, Toyota E, Suzuki K, Saito T, Fujita A, et al. Clinical evaluation of a line probe assay kit for the identification of *Mycobacterium* species and detection of drug-resistant *Mycobacterium tuberculosis*. Kekkaku. 2013 Mar;88(3):291–6.

70. Willby MJ, Wijkander M, Havumaki J, Johnson K, Werngren J, Hoffner S, et al. Detection of *Mycobacterium tuberculosis pncA* Mutations by the Nipro Genoscholar PZA-TB II Assay Compared to Conventional Sequencing. Antimicrob Agents Chemother 2018 Jan 1 [cited 2019 Dec 2];62(1). Available from: https://aac.asm.org/content/62/1/e01871-17

71. Nikolayevskyy V, Hillemann D, Richter E, Ahmed N, van der Werf MJ, Kodmon C, et al. External Quality Assessment for Tuberculosis Diagnosis and Drug Resistance in the European Union: A Five Year Multicentre Implementation Study. PloS One. 2016;11(4):e0152926.

72. Jones JM, Armstrong LR. Delayed and Unreported Drug-Susceptibility Testing Results in the US National Tuberculosis Surveillance System, 1993-2014. Public Health Rep Wash DC 1974. 2017 Aug;132(4):480–7.

73. Piersimoni C, Mustazzolu A, Giannoni F, Bornigia S, Gherardi G, Fattorini L. 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. 2013 Jan;51(1):291–4.

74. Simons SO, van Ingen J, van der Laan T, Mulder A, Dekhuijzen PNR, Boeree MJ, et al. Validation of *pncA* gene sequencing in combination with the mycobacterial growth indicator tube method to test susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J Clin Microbiol. 2012 Feb;50(2):428–34.

75. Heifets L. Susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. J Med Microbiol. 2002;51:11–2.

76. Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. J Med Microbiol. 2002 Jan;51(1):42–9.

77. Hoffner S, Angeby K, Sturegård E, Jönsson B, Johansson A, Sellin M, et al. Proficiency of drug susceptibility testing of *Mycobacterium tuberculosis* against pyrazinamide: the Swedish experience. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2013 Nov;17(11):1486–90.

78. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* and Nontuberculous Mycobacteria Drug Susceptibility Testing Program Report of Results. 2012 May. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MTBNTM.pdf

79. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Drug Susceptibility Testing Program Report of Results. 2012 Nov. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_November_2012.pdf

80. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2013 May. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_May_2013.pdf

81. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2013 Nov. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/

topic/laboratory/mpep/pdf/MPEP_November_2013.pdf

82. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2014 May. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_May_2014.pdf

83. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2014 Nov. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_November-2014_Report_WEB.pdf

84. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2015 May. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/May2015MPEP_ReportWEB.pdf

85. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2015 Nov. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/November2015MPEP_ReportWEB.pdf

86. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2016 Apr. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/april2016_mpep_reportweb.pdf

87. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2016 Aug. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/april2016_mpep_reportweb.pdf

88. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2017 Feb. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_February_2017_Final_Report.pdf

89. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2017 Aug. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP-August-2017-FinalReport.pdf

90. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2018 Feb. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_February_2018_Report.pdf

91. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2018 Aug. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_August_2018.pdf

92. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2019 Mar. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP-March-2019.pdf

93. Centers for Disease Control and Prevention. *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing Program Report of Results. 2019 Aug. Model Performance Evaluation Program (MPEP). Available from: https://www.cdc.gov/tb/topic/laboratory/mpep/pdf/MPEP_AUG2019.pdf

94. Piersimoni C, Mustazzolu A, Iacobino A, Giannoni F, Santoro G, Gherardi G, et al. Pyrazinamide susceptibility testing: proposed new standard with the BACTECTM MGIT™ 960 system. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016;20(12):1677–80.

95. Lin S-YG, Desmond E, Bonato D, Gross W, Siddiqi S. Multicenter evaluation of Bactec MGIT 960 system for second-line drug susceptibility testing of *Mycobacterium tuberculosis* complex. J Clin Microbiol. 2009 Nov;47(11):3630–4.

96. Mustazzolu A, Iacobino A, Giannoni F, Piersimoni C, Italian Multicentre Study on Resistance to Antituberculosis Drugs (SMIRA) Group, Fattorini L. Improved Bactec MGIT 960 Pyrazinamide Test Decreases Detection of False *Mycobacterium tuberculosis* Pyrazinamide Resistance. J Clin Microbiol. 2017;55(12):3552–3.

97. Morlock GP, Tyrrell FC, Baynham D, Escuyer VE, Green N, Kim Y, et al. Using Reduced Inoculum Densities of *Mycobacterium*

*tuberculosis* in MGIT Pyrazinamide Susceptibility Testing to Prevent False-Resistant Results and Improve Accuracy: A Multicenter Evaluation. Tuberc Res Treat. 2017;2017:3748163.

98. Mustazzolu A, Piersimoni C, Iacobino A, Giannoni F, Chirullo B, Fattorini L. Revisiting problems and solutions to decrease *Mycobacterium tuberculosis* pyrazinamide false resistance when using the Bactec MGIT 960 system. Ann Ist Super Sanita. 2019 Mar;55(1):51–4.

99. Werngren J, Sturegård E, Juréen P, Ängeby K, Hoffner S, Schön T. Reevaluation of the critical concentration for drug susceptibility testing of *Mycobacterium tuberculosis* against pyrazinamide using wild-type MIC distributions and *pncA* gene sequencing. Antimicrob Agents Chemother. 2012 Mar;56(3):1253–7.

100. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011 May;55(5):2032–41.

101. CRyPTIC Consortium and the 100,000 Genomes Project, Allix-Béguec C, Arandjelovic I, Bi L, Beckert P, Bonnet M, et al. Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing. N Engl J Med. 2018 11;379(15):1403–15.

102. Shea J, Halse TA, Lapierre P, Shudt M, Kohlerschmidt D, Van Roey P, et al. Comprehensive Whole-Genome Sequencing and Reporting of Drug Resistance Profiles on Clinical Cases of *Mycobacterium tuberculosis* in New York State. J Clin Microbiol. 2017;55(6):1871–82.

103. Jajou R, van der Laan T, de Zwaan R, Kamst M, Mulder A, de Neeling A, et al. WGS more accurately predicts susceptibility of *Mycobacterium tuberculosis* to first-line drugs than phenotypic testing. J Antimicrob Chemother. 2019 Sep 1;74(9):2605–16.

104. Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study. Lancet Infect Dis. 2015 Oct;15(10):1193–202.

105. Velásquez GE, Calderon RI, Mitnick CD, Becerra MC, Huang C-C, Zhang Z, et al. Pyrazinamide Resistance Assays and Two-Month Sputum Culture Status in Patients with Multidrug-Resistant Tuberculosis. Antimicrob Agents Chemother. 2016;60(11):6766–73.

106. Dormandy J, Somoskovi A, Kreiswirth BN, Driscoll JR, Ashkin D, Salfinger M. Discrepant results between pyrazinamide susceptibility testing by the reference BACTEC 460TB method and *pncA* DNA sequencing in patients infected with multidrug-resistant W-Beijing *Mycobacterium tuberculosis* strains. Chest. 2007 Feb;131(2):497–501.

107. Simons SO, van der Laan T, Mulder A, van Ingen J, Rigouts L, Dekhuijzen PNR, et al. Rapid diagnosis of pyrazinamide-resistant multidrug-resistant tuberculosis using a molecular-based diagnostic algorithm. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2014 Oct;20(10):1015–20.

108. Li H, Chen J, Zhou M, Geng X, Yu J, Wang W, et al. Rapid detection of *Mycobacterium tuberculosis* and pyrazinamide susceptibility related to *pncA* mutations in sputum specimens through an integrated gene-to-protein function approach. J Clin Microbiol. 2014 Jan;52(1):260–7.

109. Tam KK-G, Leung KS-S, Siu GK-H, Chang K-C, Wong SS-Y, Ho P-L, et al. Direct Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis* by Use of *pncA* PCR Sequencing. J Clin Microbiol. 2019 Aug;57(8).

110. Njire M, Wang N, Wang B, Tan Y, Cai X, Liu Y, et al. Pyrazinoic Acid Inhibits a Bifunctional Enzyme in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2017;61(7).

111. Dillon NA, Peterson ND, Feaga HA, Keiler KC, Baughn AD. Anti-tubercular Activity of Pyrazinamide is Independent of trans-Translation and RpsA. Sci Rep 2017 Jul 21;7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5522395/

112. Lamont EA, Dillon NA, Baughn AD. The Bewildering Antitubercular Action of Pyrazinamide. Microbiol Mol Biol Rev 2020 Mar 4; Available from: https://journals.asm.org/doi/abs/10.1128/MMBR.00070-19

113. Meehan CJ, Goig GA, Kohl TA, Verboven L, Dippenaar A, Ezewudo M, et al. Whole genome sequencing of *Mycobacterium tuberculosis*: current standards and open issues. Nat Rev Microbiol. 2019 Sep;17(9):533–45.

114. Gopal P, Grüber G, Dartois V, Dick T. Pharmacological and Molecular Mechanisms Behind the Sterilizing Activity of Pyrazinamide. Trends Pharmacol Sci. 2019 Dec;40(12):930–40.

115. Walker TM, Merker M, Kohl TA, Crook DW, Niemann S, Peto TEA. Whole genome sequencing for M/XDR tuberculosis surveillance and for resistance testing. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2017 Mar;23(3):161–6.

116. Iwamoto T, Murase Y, Yoshida S, Aono A, Kuroda M, Sekizuka T, et al. Overcoming the pitfalls of automatic interpretation of whole genome sequencing data by online tools for the prediction of pyrazinamide resistance in *Mycobacterium tuberculosis*. PloS One. 2019;14(2):e0212798.

117. Doyle RM, Burgess C, Williams R, Gorton R, Booth H, Brown J, et al. Direct Whole-Genome Sequencing of Sputum Accurately Identifies Drug-Resistant *Mycobacterium tuberculosis* Faster than MGIT Culture Sequencing. J Clin Microbiol. 2018 Aug 1;56(8):e00666-18.

118. Park J, Shin SY, Kim K, Park K, Shin S, Ihm C. Determining Genotypic Drug Resistance by Ion Semiconductor Sequencing With the Ion AmpliSeqTM TB Panel in Multidrug-Resistant *Mycobacterium tuberculosis* Isolates. Ann Lab Med. 2018 Jul;38(4):316–23.

119. Deeplex Myc-TB Technical Note.GenoScreen; 2020. Available from: https://www.genoscreen.fr/images/genoscreen-services/deeplex/technical_note_20200706_RUO.pdf

120. Makhado NA, Matabane E, Faccin M, Pinçon C, Jouet A, Boutachkourt F, et al. Outbreak of multidrug-resistant tuberculosis in South Africa undetected by WHO-endorsed commercial tests: an observational study. Lancet Infect Dis. 2018 Dec;18(12):1350–9.

121. Shi W, Chen J, Feng J, Cui P, Zhang S, Weng X, et al. Aspartate decarboxylase (PanD) as a new target of pyrazinamide in *Mycobacterium tuberculosis*. Emerg Microbes Infect. 2014 Aug;3(8):e58.

122. World Health Organization, FIND. Technical guide on next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex. World Health Organization; 2018. Report No.: WHO/CDS/TB/2018.19. Available from: http://www.who.int/tb/publications/2018/WHO_technical_guide_nextgen_sequencing/en/

123. Ängeby K, Juréen P, Kahlmeter G, Hoffner SE, Schön T. Challenging a dogma: antimicrobial susceptibility testing breakpoints for *Mycobacterium tuberculosis*. Bull World Health Organ. 2012 Sep 1;90(9):693–8.

124. Siddiqi S, Rüsch-Gerdes S. MGIT Procedure Manual. FIND. Foundation for Innovative Diagnostics; 2006. Available from: https://www.finddx.org/wp-content/uploads/2016/02/mgit_manual_nov2006.pdf

125. Becton Dickinson and Company. BD BACTEC MGIT 960 PZA Kit. 2020. Available from: https://www.bd.com/resource.aspx?IDX=18343.

## Association of Public Health Laboratories

The Association of Public Health Laboratories (APHL) works to strengthen laboratory systems serving the public's health in the US and globally. APHL's member laboratories protect the public's health by monitoring and detecting infectious and foodborne diseases, environmental contaminants, terrorist agents, genetic disorders in newborns and other diverse health threats.

This project was 100% funded with federal funds from a federal program of $1,629,896. This publication was supported by Cooperative Agreement # NU60OE000104-02 from the US Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC and the Department of Health and Human Services.


8515 Georgia Avenue, Suite 700
Silver Spring, MD 20910
Phone: 240.485.2745
Fax: 240.485.2700
www.aphl.org