Issues in *Mycobacterium tuberculosis* Complex (MTBC) Drug Susceptibility Testing: Pyrazinamide (PZA)

BACKGROUND

Pyrazinamide (PZA) is a critical component of first-line drug combination therapy for Mycobacterium tuberculosis complex (MTBC) including both susceptible and multi-drug resistant tuberculosis (MDR TB). Inclusion of PZA has shortened the previous 9–12 month chemotherapy regimen to 6 months.[1] PZA has also become an essential part of MDR TB treatment regimens that include novel compounds now clinically available, such as bedaquiline.[2,3] PZA is inactive against organisms in the growth phase during standard culture conditions at neutral pH. 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.[4–8]

The mechanism of action of PZA and resistance to PZA by *Mycobacterium tuberculosis* (MTBC) is not well understood. Pyrazinamide is a pro-drug which requires conversion to its active form of pyrazinoic acid (POA) by MTBC. Pyrazinamide enters mycobacteria by passive diffusion and is then transformed in the cytoplasm by a nicotinamidase that has pyrizinamidase (PZase) activity, encoded by the *pncA* gene of MTBC. Pyrazinoic acid accumulates in the cytoplasm and is actively expelled by a putative efflux pump. Outside of the bacilli, POA is protonated and then re-enters the organism and 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.[9,10] Recently, the ribosomal protein S1 (translated from the *rpsA* gene) was identified as a target of POA, which interferes with trans-translation activity, which is required for efficient protein synthesis.[11]

The primary mechanism of PZA resistance is due to mutations in the *pncA* gene resulting in loss of PZase activity, thus preventing conversion of PZA to POA.[12–15] In particular, mutations in specific amino acid locations in the protein affecting catalytic sites of the PZase enzyme and Fe2+ ion binding site cause loss of PZase activity and are associated with MTBC PZA resistance.[16–18]

Molecular analysis of the coding region of the *pncA* gene and promoter region from several studies has identified mutations in 46–97% of phenotypically PZA resistant isolates. It is possible that some of the phenotypically resistant isolates without identified mutations in the *pncA* gene or promoter region may actually be susceptible due to issues with phenotypic drug susceptibility testing (DST) [see *Practical Laboratory Issues*]. 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. Miotto et al. assessed *pncA* sequence variations in 1,950 clinical isolates, and

correlated them with phenotype, PZase activity, structural and phylogenetic data. Using these data, mutations were classified according to probability of predicting resistance.[19] Whitfield et al. analyzed *pncA* mutations that were not found to confer PZA resistance at the 100 ug/mL critical concentration in the MGIT 960 system (Becton-Dickinson, Sparks, MD). They observed that most of the *pncA* polymorphisms associated with susceptible isolates identified in the study had a PZA MIC between 50 and 100 ug/mL, i.e. just below the critical concentration.[20] A recently published systematic review has summarized published mutations associated with PZA resistance.[21] Loss of PZase activity does not appear to adversely affect organism viability; indeed, Aono et al. recently reported on the detection of three strains completely lacking the *pncA* gene; these strains were highly resistant to PZA, with minimum inhibitory concentrations (MIC) >1600 mg/L.[22] Additional evidence is required (e.g. MIC testing, other functional and clinical studies) to determine if the new mutation(s) truly cause phenotypic PZA resistance.[13,14,19–21,23–27]

Not all *pncA* mutations result in detectable PZA resistance by current phenotypic DST,[20] and some PZA resistant isolates may not have any *pncA* or promoter region mutations (*pncA*-WT). Resistance may 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.[9,11,28,29] *Mycobacterium canettii*, a potential precursor to *M. tuberculosis*, is naturally resistant to PZA and only contains a silent nucleotide substitution A138G (Ala46Ala) in *pncA* and no other mutations, suggesting resistance is due to other mechanisms.[28,30]

Other potential gene targets have been recently identified such as *rpsA*, and panD. To date, a few evaluations of these targets with *pncA*-WT phenotypically PZA resistant isolates have determined that while there may be a role of these gene targets in MTBC PZA resistance, it is not fully understood, and may only account for a small proportion of isolates.[10,23,26,30–33]

Pyrazinamide monoresistance is often an indicator of *Mycobacterium bovis* or *Mycobacterium bovis* BCG, which are naturally resistant to PZA. If *pncA* sequencing is performed, the mutation at nucleotide position 169, encoding for amino acid substitution of histidine to aspartic acid at position 57 in PZase (His57Asp) is observed.[15,28,34]

PRACTICAL LABORATORY ISSUES

Pyrazinamide Drug Susceptibility Testing and Test Methods

The current Clinical Laboratory Standards Institute (CLSI) considers the BACTEC 460 radiometric broth method as the reference method.[35] However, the BACTEC 460 has since been discontinued, and is no longer available. As an alternate, CLSI and the *Manual of Clinical Microbiology*[35,36] states that PZA DST may be performed using the commercial nonradiometric rapid broth systems, but that repeat testing to confirm initial resistant results should be considered. Two broth-based methods are currently FDA-cleared for use: the VersaTREK MYCO TB system (TREK Diagnostics) and the BACTEC MGIT 960 (Becton-Dickinson).

The most commonly utilized system, the MGIT 960, has been more widely evaluated than the VersaTREK MYCO TB.[37] An evaluation of the MGIT 960 system for PZA in comparison to the BACTEC 460 radiometric system found the MGIT 960 to be comparable to the BT 460.[38]

However, since the introduction of the MGIT 960, several issues with susceptibility testing have been noted, in particular, difficulties with reproducibility, particularly with PZA.[23,39–41]

The MGIT 960 utilizes an acidified pH (approximately 5.9), Middlebrook 7H9 broth modified with growth supplement (bovine albumin, dextrose and polyoxyethylene stearate) and a modified proportion method, with the critical concentration of 100 mg/L of PZA. The system has continuous automated monitoring in which growth is detected using a non-radiometric method that detects consumption of oxygen by a fluorescence indicator.

The VersaTREK MYCO TB system utilizes an acidified medium (Middlebrook 7H9 based, pH 5.9 – 6.0) with growth supplement (MYCO GS) and a critical concentration of 300 mg/L of PZA. Pressure changes within the culture bottle are used to detect growth, and these pressure changes are presented as a growth curve; growth is demonstrated by a gas consumption curve (as MTBC grows it consumes oxygen) downward. The VersaTREK system has continuous automated monitoring.

A microtitre plate broth dilution system, Sensititre (TREK Diagnostic Systems, Cleveland, OH) has been developed for first and second line antituberculosis drugs. However PZA DST requires a more acidic medium; therefore PZA is not in the panel of available drugs.

Other methods that have not been cleared by the FDA have been developed and assessed for PZA. These include the resazurin microtitre assay (REMA), colorimetric nitrate reductase assay, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reduction test).[26,42–46] The REMA and MTT assays detect bacterial growth in a microtitre well format through redox reactions. The nitrate reduction assay also requires growth in liquid or solid media. In the US, it is not known if any laboratories are employing these methods for PZA DST (D. Warshauer, personal communication, August 4, 2014).

The PZase test, which detects the production of pyrazinamidase by the organism and it's ability to convert PZA to POA (not FDA-cleared), requires a sufficient inoculum with a large number of organisms in order to detect PZase activity. The assay can be misinterpreted resulting in false resistance. Some isolates with PZase activity have been determined to be PZA resistant (resistance other than a mutation affecting PZase). Pyrizinamidase assay sensitivity has been reported to vary between 79 – 96%.[12,25,26,40,47] The PZase test detects deamidation of PZA to pyrazinoic acid and ammonia through formation of a pink band in the subsurface agar that diffuses into the medium indicating enzymatic hydrolysis of PZA to free pyrazinoic acid. Mestdagh et al. found that 39/42 PZase positive isolates were determined to be PZA susceptible by the BACTEC 460, confirming other findings that not all PZA resistant isolates are PZase negative.[48] They concluded that negative PZase results could be used to determine PZA resistance, but care should be taken in the interpretation of PZase positive results.[48–50] Further studies should be undertaken and are needed to better establish the relationship between phenotypic DST and PZase testing.

Molecular methods include *pncA* gene sequencing to identify mutations. As described earlier, some *pncA* mutations have a high association with phenotypic resistance, but not all.[19–21] Line Probe Assay (LiPA) has been developed for the detection of mutations within the genetic loci associated with resistance to pyrazinamide (*pncA*), but is not FDA cleared for use.[51]

Reproducibility of Susceptibility Testing for PZA

In-vitro testing of PZA is difficult and generally it is unreliable. The MGIT 960 has known issues with specificity (false resistance), and even when repeat testing is performed (as recommended by CLSI M24-2A),[35] false resistance can still occur.[40,52,53] The bactericidal activity of PZA is optimal only in an acid environment (pH 5.5 – 5.6), and is almost inactive at neutral pH. Additionally, the acid environment itself inhibits the growth of MTBC isolates. Therefore, the MIC of PZA is pH dependent and has been shown to increase with increasing pH. It has been demonstrated that at a pH of 5.5, the MIC was 50 mg/L, whereas at a pH of 5.8 the MIC was 100 mg/L, and at a pH of 6.1, the MIC was 200 mg/mL due to enhanced viability in more alkaline media.[6,54,55] The drug concentration used for PZA testing in the liquid broth systems (100 mg/L) (MGIT960) is greater than that used for conventional solid media DST (pH 5.5) as well as the expected concentration in serum and tissue, in order to compensate for the higher pH.[6]

The reproducibility of PZA phenotypic DST is particularly affected by the size of the test inoculum. A large inoculum (10^7 cells/ml to 10^8 cells/ml) has been shown to increase the pH to 7, inactivating PZA and resulting in false resistance. Only a small increase in pH (less than 0.3 units) was found with an inoculum containing 10^6 cells/mL, the most commonly used inoculum for DST. Phenotypic 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 increase pH of acidic media and binds to POA, which may result in false resistance.[8,55,56] Aono et. Al. showed that phenotypic DST results may reflect a mixture of strains with differing properties.[22] Additionally, 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 readily identified by poor growth in the control tube as well as the PZA tube, rendering the result invalid.

Proficiency Testing

Proficiency testing is not readily available and is not included as one of the World Health Organization Network of Supranational Reference Laboratories yearly proficiency test panels. In the US, the College of American Pathologists provides proficiency testing for PZA and other antituberculosis drugs; however, their program provides only two challenge isolates per year and includes only pan-susceptible strains. The Centers for Disease Control and Prevention (CDC) offers a performance evaluation program for MTBC DST twice a year that includes five isolates that may be pan-susceptible or resistant to various drugs. The CDC program is not a formal, graded proficiency testing program, but is an excellent self-assessment tool for laboratories performing MTBC DST and an opportunity to compare results to those obtained by other participants using the same methods.

Table 1. Culture-based and Molecular Drug Susceptibility Testing Methods

	Culture-based Method	Determination of Resistance
FDA Cleared	MGIT 960	Growth in the presence of PZA at the critical concentration of 100 mg/L
	VersaTREK MYCO TB	Growth in the presence of PZA at the critical concentration of 300 mg/L
Research Use Only (RUO)	PZase**	Lack of PZase activity

	Molecular-based Method	Determination of Resistance
Research Use Only (RUO)	Sanger Sequencing	Detection of mutations within the genetic loci and promoter region associated with resistance to pyrazinamide (*pncA*).

Shaded cells refer to non-phenotypic DST methods

*Solid media proportion methods (Lowenstein-Jensen or 7H10 agar based) have been used or proposed, but are slow and labor-intensive, and not recommended for PZA DST [35,54,57–59]

** not FDA cleared

There is a lack of quality data upon which to base development and implementation priorities for new drug treatment regimens that include PZA.[60,61] 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.[60] False resistant PZA DST results were observed for two PZA susceptible MTBC strains in the 2012 CDC model performance evaluation program (MPEP) for MTBC drug susceptibility testing. For one susceptible strain 33 of 70 (47%) laboratories reported resistance and for the second strain, 16 of 74 (22%) laboratories reported resistance.[62] This false resistance with the MGIT 960 system continued to be seen in the 2013 program. In the National Microbiology Laboratory 2013 Proficiency Testing Program administered by the Canadian National Reference Centre for Mycobacteriology (National Microbiology Laboratory, Public Health Agency of Canada), one out of the five participating laboratories reported false susceptible and false resistance for PZA DST (F. Jamieson, personal communication).

Approaches to Improving Reproducibility and Specificity Using the MGIT 960

PZA DST Inoculum:

Lack of standardization of test inoculum significantly affects results. The manufacturer's recommended method for testing using MGIT 960 likely results in differences in the amount of organism used in testing. The concentration of MTBC in the MGIT 960 compared to the Bactec 460 is greater than 2.5 times, and there is variation in the concentration of the inoculum used in the MGIT 960, depending on the day of test set-up. If a much larger inoculum or non-homogenous inoculum (clumps) is used for testing, there is a high potential for false resistance.[40,52,60]

In order to ensure a comparable, uniform inoculum for each test, 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 960 tube, removing supernatant and then diluting to a 0.5 McFarland standard.[63] In a study by Piersimoni et al., they recommend using a reduced inoculum of 0.25 mL from the positive MGIT tube instead of 0.5 mL, as is recommended by the manufacturer, for repeat testing. They found improved agreement with the BACTEC 460, used as the gold standard in this study.[52] There are very few data to determine if this is an issue with the VersaTREK system.[37]

Use of more than one test concentration or determination of MICs:

Due to normal variation in set-up, the present critical concentration used for PZA (100 mg/L) 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 has to rely on using a distribution of MICs from wild-type MTBC strains, as clinical outcome data for treatment with PZA alone is not obtainable. The definition of the critical concentration for PZA categorizes up to 10% of wild-type MTBC strains as drug resistant. The World Health Organization has stated that the critical concentration defining resistance is often very close to the MIC required to achieve antimycobacterial activity, increasing the probability of misclassification of susceptibility or resistance and leading to poor reproducibility of DST results.[64]

Varying interpretations of results have been suggested, including a cutoff of 300 mg/L, a range (100, 300 and 900 corresponding to susceptible, intermediate and resistant) from radiometric data, or setting the critical concentration at 200 mg/L based on the theoretical MIC at a pH of 6.0.[55,56] Alternatively, Werngren et al. suggested interpreting MICs of less than 64 mg/L as susceptible, less than 128 mg/L as intermediate and greater than 128 mg/L as resistant based on a study of phenotypic resistance and *pncA* gene mutations in PZA resistant MTBC.[65] There is a need to further characterize the phenotype of isolates with specific *pncA* mutations and their correlation to MICs.[20,24] Aono et al. recently reported the detection of sub-populations within strains that exhibited differing characteristics affecting initial PZA DST results. As noted earlier, they also identified three isolates where the *pncA* gene was deleted, with phenotypic resistance to PZA.[22]

IMPACT ON CLINICAL OUTCOMES

Pyrazinamide is a critical component of first-line drug combination therapy for tuberculosis for both susceptible and multi-drug resistant tuberculosis (MDR TB). Inclusion of PZA in the first two months of treatment has shortened the previous 9–12 month chemotherapy regimen to six months.[1]

World Health Organization guidelines have determined that PZA should be included in the intensive phase of the treatment regimens for MDR TB.[1,66,67] Pyrazinamide is also considered an important component of shorter, new drug regimens, as well as in combination with novel antituberculosis drugs recently developed, such as PA-824, a nitroimidazo-oxazine, with moxifloxacin and PZA, and bedaquiline.[2,3]

As PZA is typically used for a short amount of time (i.e. the first two months of the six month standard US-CDC treatment regimen), it is important that the DST results are provided rapidly, accurately, and reliably. False resistance may result in unnecessarily prolonged therapy.

The lack of reliable PZA resistance data hampers the efforts for determining priorities of new tuberculosis treatment regimens and determining the effectiveness of drug treatments using novel drugs.

AREAS OF ONGOING RESEARCH

pncA and other gene target molecular assays:

Mutations of the *pncA* gene have been identified along the entire length of the gene, as well as in the putative upstream regulatory region, for approximately 700 base pairs. There are a large number of *pncA* mutations that have been published, but there are no predominant mutations. Some have been identified as always being associated with resistance to PZA, but more studies are needed to determine the phenotype of *pncA* mutations.[19–21,24]

The use of *pncA* sequencing for PZA susceptibility was suggested by Dormandy et al. in an analysis of MDR TB isolates which appeared phenotypically susceptible by BACTEC 460 but harbored a specific *pncA* mutation with increased MICs.[68] Simons, et al. have proposed incorporating *pncA* sequencing with phenotypic DST as part of the clinical testing algorithm. They found a sensitivity of 73% for non-synonymous *pncA* mutations and a specificity of 100%, using an algorithm of *pncA* gene sequence analysis in all isolates found to be PZA resistance by MGIT 960.[53] A systematic review and meta-analysis of pyrazinamide drug susceptibility testing found that *pncA* gene sequencing could be used to potentially rule out PZA resistance in a low prevalence, non-MDR TB setting. Furthermore, in high prevalence settings *pncA* mutations may predict true PZA resistance.[25] Simons, et al. found that *pncA* sequence analysis with rpoB mutation analysis for the detection of PZA resistant MDR-TB had a sensitivity and specificity of 96.8% and 94.2% respectively, with a positive predictive value of 90.9% and negative predictive value of 98.0%.[21] Most recently, a large systematic review evaluated the utility of mutations in PZase (*pncA*) and the putative upstream regulatory (promoter) region for *pncA* for the molecular diagnosis of PZA resistance in MTBC. Of 2760 PZA resistant MTBC isolates, 83% had at least one mutation in *pncA* and/or the upstream regulatory region, and of 3329 PZA sensitive isolates, 9% had a mutation in *pncA*. Sensitivity and specificity based on mutations from isolates with phenotypic resistance determined by either the BACTEC 460 or MGIT 960 was found to be 80% and 91%, respectively.[21] Similarly, Miotto et al. found almost 85% of genetic variants identified in the *pncA* gene were associated with phenotypic resistance to PZA and they were able to classify them as “high-confidence” resistance mutations.[19] Another approach is the detection of *pncA* wild-type gene, to rule-out resistance. However, silent mutations preventing hybridization and detection would present a concern for reporting false resistance.[13]

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 pyrazinamidase, based on the *pncA* gene sequence of the isolate. This assay is useful for PZA resistance that is due to *pncA* mutations, but cannot detect resistance due to other mechanisms.[46,69]

Other gene targets identified as possible contributors to PZA resistance include *rpsA* and *panD*. The *rpsA* gene encodes ribosomal protein S1 (RpsA), a target of PZA. RpsA is involved in trans-translation, a component of the degradation process of potentially toxic protein products formed in stressed bacteria. This function is important for persister survival.[10,11,33]

The *panD* gene, which encodes aspartate alpha-decarboxylase has also been identified as a potential target by whole genome sequencing. It has been shown to be critical for survival and persistence of *M. tuberculosis in vivo*, and is involved in the pantothenate and Coenzyme A (CoA) synthesis pathway, required for cellular metabolism. It is hypothesized that PZA may inhibit pantothenate and CoA synthesis, interfering with important metabolic functions of the cell. Whole genome sequencing approaches combined with expression and functional genomics may provide additional gene targets involved in PZA resistance.[10]

Critical concentration revision and determination of breakpoints:

Research challenging the current critical concentration testing protocols, and the development of new testing breakpoints is on-going. Further determination of the true distribution of MICs in wild-type MTBC and the relationship to clinical outcomes is needed.[64,65]

Assessment of POA:

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.[9,29]

GUIDANCE

Laboratories should consider the following:

- Use fresh cultures for preparation of test inoculum for phenotypic broth based DST
- Ensure a standard inoculum for DST; Laboratories may consider validating method for inoculum preparation[40,63]
- For a MGIT 960 positive MTBC culture inoculated to the PZA test consider the following if using the MGIT 960 system (based on options in the package insert):

  1. Use a day 1 culture rather than day 2

  OR

  2. Use a day 3 culture diluted 1:5, rather than day 4 or 5 (K. Jost, personal communication, August 4, 2014)

- Repeat DST if resistant, using a day 3 culture
- All mono-resistant 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)
- Consider repeating resistant results with a reduced inoculum[40,52]
- Test all phenotypic resistant isolates by *pncA* sequence analysis
  - Consider repeat testing at different breakpoints for *pncA* isolates with mutations not previously recognized.
  - Consider any isolates with non-synonymous *pncA* gene mutations as “PZA resistant”
  - Repeat phenotypic DST for any isolates with synonymous *pncA* mutations or wild type (WT)[53]

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