April 29, 2024

Dear TB Drug Susceptibility Testing Reference Center Submitters,

Effective 05/06/2024, the National PHL Drug Susceptibility Testing Reference Center for *Mycobacterium tuberculosis* (the CDPH Microbial Diseases Laboratory (MDL)) **will be replacing routine phenotypic DST (pDST) for pyrazinamide (PZA) with whole genome sequencing (WGS) for molecular DST (mDST) of PZA as the primary test method**. This DST method replacement will be ongoing with no end date due to recurring issues with PZA pDST that may result in erroneous reports of resistance and substantial delays in final result reporting. This change is being made to avoid overreporting PZA resistance, and because MDL currently offers a reliable alternative method of determining PZA resistance via molecular testing.

This change does not affect phenotypic DST services for any other drugs.

This switch to WGS mDST as the primary test method for PZA will reduce false-resistant interpretations that may be encountered with the current phenotypic assay. MDL's WGS validation data show that mDST accurately detects resistance-conferring mutations in the pncA gene target, and that results correlate well with phenotypic resistance with both 100% diagnostic specificity and positive predictive value (PPV). For more information, please refer to the attached FAQs.

Isolates that have already been received by MDL for phenotypic PZA DST prior to this notification will undergo WGS regardless of the test ordered. Resistant pDST results for PZA will be reported only if confirmed by WGS. Results that are not confirmed by WGS will be reported as "Indeterminate" with a comment describing this final interpretation.

For new isolates submitted for first-line pDST as of 05/06/2024, PZA testing will be performed by WGS and results will be reported as soon as available (TAT 10-14 days). Phenotypic DST for PZA will only be performed as a reflex test if WGS detects an "uncertain" mutation and reported as described in our FAQ and workflow diagram.

We appreciate your patience as we implement this change; we will continue to investigate phenotypic PZA technical issues internally and with the reagent manufacturer. Please reach out to the TB DST laboratory supervisors if you have any questions or require further instruction for your submissions: Dr. Matthew Sylvester (Matthew.Sylvester@cdph.ca.gov) and Dr. Varvara Kozyreva (Varvara.Kozyreva@cdph.ca.gov).

Sincerely,

Zenda L. Berrada, PhD, PHLD(ABB), D(ABMM)
Chief: Microbial Diseases Laboratory Branch
Email: Zenda.Berrada@cdph.ca.gov
Phone: 510.307.8547

Sarah Buss PhD, D(ABMM)
Manager: HIV, Viral Hepatitis, STD, and TB
Email: sarah.buss@aphl.org
Phone: 240.485.3901

Angela M. Starks, PhD
Chief: Laboratory Branch, Division of Tuberculosis Elimination, CDC
Email: astarks@cdc.gov
Phone: 404.639.3205

FAQs:

1) **What is causing the current issues with the phenotypic PZA assay?**

Since late 2023, MDL and other laboratories around the country have experienced intermittent issues with an increase in false PZA resistance when DST is performed using the BD BACTEC MGIT testing system. Although most isolates are susceptible to PZA via MGIT DST, when PZA resistance is encountered, PZA reporting has been delayed due to intermittent QC failures, the need for repeat testing, and an increased number of non-*M. bovis* PZA-monoresistant samples, suggestive of false resistance.

2) **What evidence supports use of *pncA* gene sequencing results in lieu of phenotypic susceptibility testing for PZA?**

- The positive predictive value for *pncA* sequencing is high[1,2]; i.e., detected resistance-conferring mutations highly correlate with resistant phenotype.
- The negative predictive value for *pncA* sequencing is high for non-MDR TB isolates[1]; i.e., absence of resistance-conferring mutations in *pncA* correlates well with phenotypic susceptibility. Even though we cannot exclude the possibility of a mutation conferring PZA resistance outside of *pncA,* it is not common. Considering the low pretest probability for PZA resistance in non-MDR samples, the risk of PZA false-susceptibility in the WGS assay is much smaller than the risk associated with an increase in PZA false-resistance that is currently being observed with phenotypic DST.

3) **What is the sensitivity and specificity for prediction of resistance based on the *pncA* sequence as compared to phenotypic DST?**

Estimates of sensitivity and specificity for PZA resistance prediction based on the presence of resistant mutations in the pncA gene vary among study populations depending on the abundance of MDR and lineage 1 strains in the dataset, which have an elevated MIC close to the critical concentration[6] and other factors. Below are some numbers from different sources:

	All isolates		MDR isolates only	
Source	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
APHL[1] (= WHO 2021)	72.3%	98.8%	N/A	N/A
CDC[3]	51.9%	99.7%	95.5%	94.2%
WHO 2023[4]	78.0%	97.9%	N/A	N/A
MDL WGS validation study (n=189)	61.9%	100%	N/A	N/A
Chang *et al*, 2011[5]	85%	88%	N/A	N/A

It is important to note that the low sensitivity of sequencing-based resistance prediction as compared to phenotypic DST for PZA is likely an artifact of the low reproducibility and propensity for false-resistance of the phenotypic DST assay. Therefore, the true sensitivity of sequencing-based resistance prediction may be underestimated.

Notably, in a meta-analysis performed by Chang *et al*[5], the negative predictive value (NPV) of *pncA* sequencing for non-MDR *M. tuberculosis* was >99% which strongly supports use of *pncA* sequencing for ruling out PZA resistance.

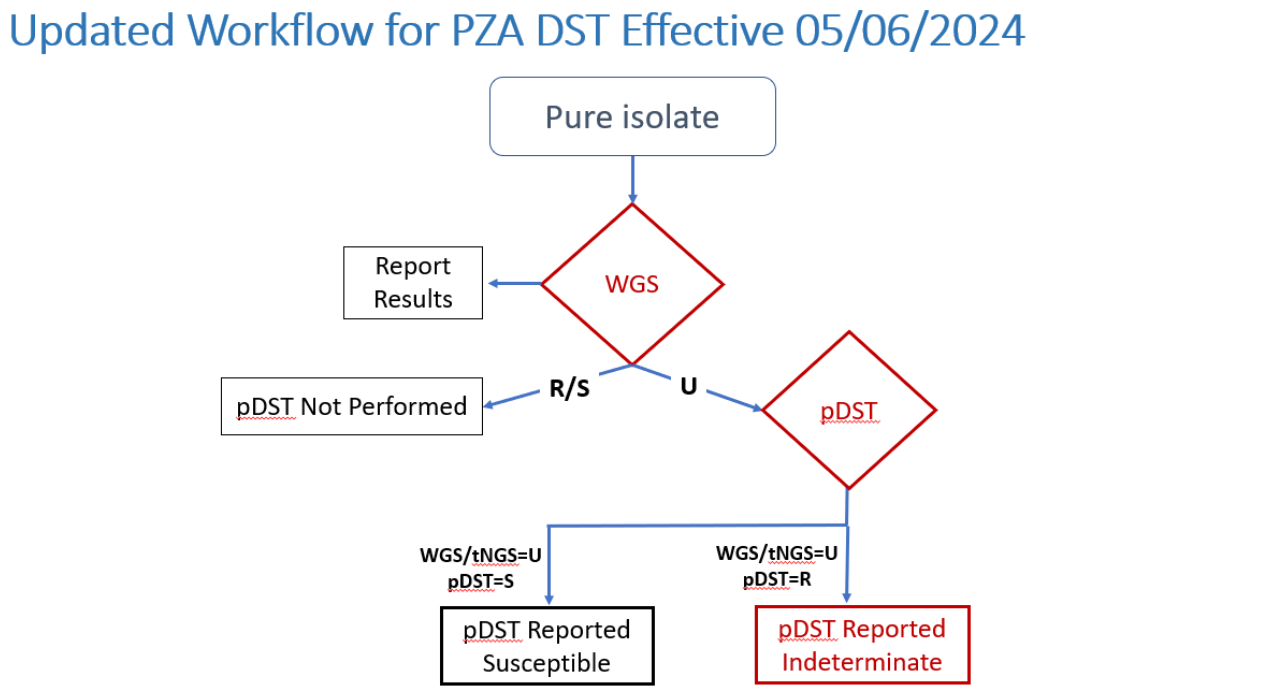
The NPV for *pncA* sequencing for MDR-TB can be estimated based on the sensitivity and specificity values for MDR isolates provided by the CDC[3] and prevalence values of MDR-TB for PZA resistance from two meta-analyses, as follows:

Meta-analysis	Prevalence	NPV
Chang *et al*, 2011[5]	51%	95%
Whitfield et al, 2015[7]	61%	93%

Considering that the NPV of *pncA* in MDR isolates has been estimated at >90%, the absence of a resistance-conferring mutation in *pncA* is a good predictor of PZA susceptibility in MDR strains.

4) **What does the modified reporting workflow look like for WGS and pDST?**
   - For isolates submitted for first-line pDST on and after 05/06/2024, PZA pDST will NOT be set up by default and WGS will be performed as the primary test. WGS results will be reported as soon as available (TAT 7 – 14 days). Other first-line drugs will be set up for pDST as usual, if ordered.
   - If the WGS result for PZA is "**No mutations associated with resistance to pyrazinamide detected**", pDST confirmation will NOT be performed due to the higher likelihood of false-resistant results.
   - If the WGS result for PZA is "**Mutation(s) associated with resistance to pyrazinamide detected**", pDST confirmation will NOT be performed. High confidence mutations in pncA that are associated with resistance are good predictors of PZA resistance.
   - If the WGS result for PZA is "**The detected mutation(s) have uncertain significance. Resistance to pyrazinamide cannot be ruled out**", pDST will be performed by reflex. If an isolate with an "Uncertain" mutation test appears to be resistant by pDST, the final interpretation for the PZA pDST result will be reported as "Indeterminate". Since we are confident in pDST susceptible results, susceptible results will be reported.

   The PZA DST workflow is summarized below:



tNGS may be available if we previously performed testing on a sediment or mixed culture with the same submitter accession.

5) **We received an "Indeterminate" phenotypic PZA result. What does this mean and what are my next steps?**

A final "Indeterminate" interpretation for pDST is made under two scenarios:
• For an uncertain WGS pncA result followed by a resistant pDST result after reflex testing, the following interpretive comment will be added to the report:
"This isolate has tested PZA-resistant using the BACTEC MGIT platform; however, the pncA mutation(s) detected by the WGS assay and reported on XX/XX/XXXX have uncertain significance. The effect of this pncA mutation is unknown and PZA resistance cannot be confirmed by phenotypic DST due to assay variability combined with strain characteristics such as strain lineage or unknown factors which could lead to phenotypic resistance."

• For a phenotypic PZA resistant result and no pncA mutation detected by WGS, the following interpretive comment will be added to the report (applicable only for samples that were received prior implementation of the new workflow; See FAQ #6 below):

"This isolate has tested PZA-resistant using the BACTEC MGIT platform; however, NO pncA mutations known to be associated with resistance were detected by the WGS assay as reported on XX/XX/2024. These discordant results may be caused by phenotypic DST assay variability combined with strain characteristics such as strain lineage or unknown factors which could lead to phenotypic resistance."

As a next step for additional clinical guidance, consult your state TB program (https://www.cdc.gov/tb/links/tboffices.htm) or the TB Centers of Excellence (TB COE, 877-390-6682, https://www.cdc.gov/tb/education/tb_coe/default.htm)

6) **Will phenotypic DST for PZA still be performed for isolates submitted prior to this announcement?**

Yes, however, susceptible/resistant phenotypic PZA results will only be reported in the following two scenarios:

a) When phenotypic PZA results are "susceptible";
b) When phenotypic PZA results are "resistant" and high-confidence resistance mutations are detected in *pncA* by WGS.

If the WGS-based PZA result states "No mutations associated with resistance to pyrazinamide detected" or "The detected mutation(s) have uncertain significance", and phenotypic PZA result is "Resistant", the final interpretation will be reported as "Indeterminate" due to higher likelihood of phenotypic false-resistance.

References:

[1]https://www.aphl.org/aboutAPHL/publications/Documents/ID-2022-MTBC-DST-Pyrazinamide.pdf

[2]Rodwell, T., P. Miotto, C. Köser, T. Walker, P. W. Fowler, J. Knaggs, Z. Iqbal et al. "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance." (2021).

[3]CDC personal communications.

[4]Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance, Second edition, WHO, 2023. https://www.who.int/publications/i/item/9789240082410

[5]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. doi: 10.1128/AAC.00630-11. Epub 2011 Jul 18. PMID: 21768515; PMCID: PMC3186960.

[6]Mok S, Roycroft E, Flanagan PR, Montgomery L, Borroni E, Rogers TR, Fitzgibbon MM, 2021. Overcoming the Challenges of Pyrazinamide Susceptibility Testing in Clinical Mycobacterium tuberculosis Isolates. Antimicrob Agents Chemother 65:10.1128/aac.02617-20.

[7]Whitfield, Michael G., Heidi M. Soeters, Robin M. Warren, Talita York, Samantha L. Sampson, Elizabeth M. Streicher, Paul D. Van Helden, and Annelies Van Rie. "A global perspective on pyrazinamide resistance: systematic review and meta-analysis." PloS one 10, no. 7 (2015): e0133869.