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# Characterization of novel *Mycobacterium tuberculosis pncA* gene mutations in clinical isolates from the Ukraine

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ABSTRACT

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* cases in the Ukraine are increasing. Pyrazinamide (PZA) is critically important for first- and second-line tuberculosis (TB) treatment regimes. However, PZA drug susceptibility testing is time consuming and technically challenging. The present study utilized Next-generation sequencing (NGS) to identify mutations in the *pncA* gene from clinical isolates and to assess the prevalence of *pncA* gene mutations in MDR/XDR-TB patients. Clinical isolates were inactivated in molecular transport media and shipped from Kharkiv, Ukraine, to San Antonio, TX. Whole-genome and targeted *pncA* gene sequencing was carried out using Illumina MiSeq instrumentation. Mutations were noted in 67 of 91 (74%) clinical isolates comprising substitutions, insertions, and deletions in the *pncA* coding and upstream promoter region. Of 45 mutation types, there were 11 novel, i.e., to date unknown, *pncA* mutations identified of which 3 were confirmed PZA resistant. Seven isolates contained mixed base mutations, whereas 4 harbored doubled mutations. Data reported here further support use of NGS for *pncA* gene characterization and may contribute in significant fashion to PZA therapy, especially in MDR- and XDR-TB patients.


## 1. Introduction

An alarming increase in multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) cases in the Ukraine continues to challenge effective patient treatment efforts (Pavlenko et al., 2018). The World Health Organization (WHO) recommends inclusion of pyrazinamide (PZA) in standard short-course TB treatment regimens as well as aggressive treatment of patients with MDR and XDR-TB (Falzon et al., 2017; World Health Organization (WHO), 2016a). The synergistic antimicrobial effect of PZA in concert with rifampin (RIF), isoniazid, and fluoroquinolones has made PZA an essential drug in effective MDR-TB treatment.

However, PZA-resistant cases particularly among MDR and XDR patients have been reported worldwide (Allana et al., 2017; Kurbatova et al., 2013; Liu et al., 2017; Ramirez-busby and Faramarz, 2015). The use of standard PZA resistance detection by phenotypic drug susceptibility testing (DST) in *Mycobacterium tuberculosis* (MTB) is problematic due to pH restraints, inconsistencies in established critical concentrations, and long turnaround times, e.g., days to weeks (Chedore et al., 2010a; Zhang et al., 2002). Furthermore, DST results are often misleading due to false positives, e.g., detection of resistance in PZA-sensitive samples (Zhang et al., 2002). The primary mechanism for PZA resistance is attributed to high mutational diversity in pyrazinamidase, an enzyme encoded by the *pncA* gene. Resistance-conferring mutations include several previously confirmed single nucleotide polymorphisms, insertions, deletions, and frameshifts that span the entire 561–base pair coding region as well as the upstream promoter.

For effective TB treatment, it is crucially important to rapidly identify 1) those patients who could benefit from a PZA-containing regimen and 2) those for whom inclusion of PZA would not be effective. This distinction requires rapid nucleic acid–based detection approaches for determination of PZA resistance. However, due to genetic diversity in the *pncA* gene (Ramirez-busby and Faramarz, 2015), there are no commercially available nucleic acid–based tests for detection of PZA resistance. In contrast to Sanger sequencing, next-generation sequencing (NGS) using whole-genome sequencing (WGS) and/or targeted gene sequencing (TGS) enables multiplexing (indexing) multiple patient samples with considerable nucleotide coverage depth, permitting detection of mixed-strain mutations conferring PZA heteroresistance, i.e., strains containing both drug-resistant and drug-sensitive (wild-type) mutations. Thus, the rapid nature of NGS could afford timely detection of PZA resistance and improve patient care.

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The Ukraine ranks among the top 20 countries with high MDR-TB burdens in the world (World Health Organization (WHO), 2016b). According to phenotype analysis, frequencies of PZA resistance in MDR isolates can be greater than 60% (Huy et al., 2017; Pang et al., 2017; Whitfield et al., 2015). However, only a few studies have focused on genetic diversity of resistance mutations in the *pncA* gene of clinical isolates from the Ukraine. Using NGS, we have carried out genetic analysis to 1) characterize *pncA* gene mutations in MTB clinical isolates; 2) assess the prevalence of *pncA* gene mutation among TB patients presenting with MDR and XDR-TB; and 3) in a select group of isolates, compare *pncA* mutations to results obtained using phenotypic DST.

## 2. Materials and methods

### 2.1. Patient enrollment and demographics

The results reported here are part of a larger TB study of patients from 2 in-patient TB clinics in Kharkiv and 1 prison hospital in Pokrovske, Ukraine. Patient sputum was collected between March and August 2016 from individuals residing in 15 geographically diverse oblasts of Ukraine and Crimea. The majority of samples in this study ($N = 98$) were obtained from patients residing in Kharkiv ($N = 56$), Ukraine's second largest city. Additionally, 7 patients were from Luhansk, 6 from Kiev, 3 from Sumy, and 3 from Crimea. The remaining 23 patients resided in 11 other oblasts. Of the 98 clinical samples collected, 7 samples rendered inadequate sequence data upon analysis, leaving 91 patients reported in this study (73 males and 18 females; mean age of 42 years). Fifty-two (57%) were current or previously incarcerated prisoners. A total of 58 (64%) individuals were previously diagnosed with TB, i.e., documented reinfection, with 84 (92%) being chronic smokers, and 14 (15%) testing positive for HIV. Detailed patient descriptions including response to therapy and survival status will be reported separately. All patients in this study provided informed consent and were treated according to standard care. The study protocol was reviewed and approved by the Ukrainian Institutional Bioethics Commission (Kharkiv Medical Academy of Postgraduate Education).

### 2.2. Ukraine clinical isolates

Clinical isolates were obtained from either smear or Xpert® MTB/RIF-positive (Cepheid Inc., Sunnyvale, CA) tuberculosis patients or those meeting clinical criteria, i.e., physical examination, prior history of confirmed TB, or positive chest x-ray. Sputum specimens ($N = 98$) were cultured, and a selection of isolates was tested for drug susceptibility using the MGIT 960 System (BD Diagnostic Systems, NJ) with phenotypic resistance determination performed according to standard protocols as previous described (Global TB Programme, 2012; World Health Organization (WHO), 2008). For determination of drug susceptibility, the PZA critical concentration used was 100 μg/mL.

### 2.3. DNA preparation and qPCR detection

Clinical isolates were processed/analyzed in blind fashion throughout this study. After MGIT 960 DST, approximately 0.5 mL was transferred to cryotubes containing 1.5 mL PrimeStore Molecular Transport Medium® (PS-MTM; Longhorn Vaccines & Diagnostics, San Antonio, TX). PS-MTM–treated clinical isolates were transported from Kharkiv, Ukraine, to San Antonio, TX, at ambient temperature (4 days), and stored at 4 °C until analyzed.

Total nucleic acid from 200 μL PS-MTM–treated clinical isolate was extracted using PrimeXtract according to manufacturer's instructions (Longhorn Vaccines and Diagnostics, San Antonio, TX). Quantitative, real-time PCR (qPCR) was performed using PrimeMix® Multiplex MTB Detection Blend (PM-PCR; Longhorn Vaccines and Diagnostics, San Antonio, TX) and an ABI 7500 instrument (Thermofischer, Waltham, MA). For MTB detection, extracted DNA (5 μL) was added to 15 μL PM-PCR solution per manufacturer's instructions (Longhorn Vaccines and Diagnostics, San Antonio, TX). qPCR was performed in duplicate, and average cycle threshold ($C_T$) values were used to assess the initial concentration of MTB from total nucleic acid extractions.

### 2.4. WGS

Clinical isolate DNA (1–5 ng) was processed using the Nextera XT Sample Prep Kit (Illumina, San Diego, CA) and pooled using the Nextera XT Index Kit (Illumina, San Diego, CA). WGS was performed per manufacturer's instructions (Illumina, San Diego, CA) using a MiSeq 600 cycle Reagent Kit (V3). For WGS, a total of 24 clinical samples were indexed for each run to produce average base pair (bp) read lengths of ~240 bases (>500,000 reads per sample) and >30-fold coverage depth. Of the 98 clinical samples, 75 isolates (77%) analyzed by WGS produced adequate genomic data, whereas 23 rendered poor derived whole-genomic, i.e., <30× coverage depth, and/or poor quality sequence.

### 2.5. TGS

The remaining 23 clinical isolates not rendering quality WGS data were subjected to targeted *pncA* gene amplification. TGS was performed on samples with initial MTB qPCR cycle threshold values greater than 30 ($C_T > 30$). Amplification of the *pncA* gene was carried out according to manufacturer's recommendations in a 1× amplification solution containing an optimized blend of buffers, salts, and enzyme (PrimeSeq *pncA*; Longhorn Vaccines and Diagnostics, San Antonio, TX). The forward and reverse primer sequences used are 5′-ATGGACCCTATATCTG TGGCT-3′ and 5′-AACAGTTCATCCCGGTTCGG-3′, respectively. The resulting amplicon (663-bp oligonucleotide) contained the complete *pncA* gene (561 bp) and upstream promoter region. PCR amplification was carried out using an ABI 2720 Thermocycler Instrument with the following parameters: a hot start (one 2-min cycle at 96 °C) followed by 40 cycles of 15 s at 96 °C, 15 s at 55 °C, and 1 min at 72 °C. Final extension was carried out at 72 °C for 5 min. Prior to library preparation, PCR amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) per manufacturer's instructions. Purified 'amplicon' DNA (1–5 ng) was processed using a Nextera XT Sample Prep Kit (Illumina, San Diego, CA) and pooled using the Nextera XT Index Kit (Illumina, San Diego, CA). NGS was performed per manufacturer's instructions (Illumina, San Diego, CA) using a 300-cycle MiSeq Reagent Nano Kit. (Illumina, San Diego, CA). For targeted sequencing, 23 samples were indexed to produce average bp read lengths of approximately 240 bases constituting ≥150× coverage depth. A positive MTB H37Rv control sample consisting of the *pncA* 'wild-type' gene sequence was included in the analysis.

For bioinformatic analyses, sequences were assembled into 'contigs' and mapped to the *M. tuberculosis* H37Rv genome (GenBank accession number NC_000962.3) using SeqMan NGen (V8) and LaserGene (V13) Core Suite (DNAStar Inc., Madison, WI). Sequencing data were filtered to remove low-quality reads and Illumina adaptor sequences. For WGS, at least 30 nucleotide bases per locus were evaluated for validity and mutation detection using SeqMan Pro (DNAStar Inc., Madison, WI). For TGS, a minimum of 150 bases per locus was analyzed. Additionally, mutational analysis was confirmed using Exatype software (Hyrax Biosciences, Cape Town, South Africa).

## 3. Results

Of 98 clinical isolates, NGS generated acceptable sequence data for 75 using whole-genome sequencing. Those isolates rendering inadequate whole-genome sequences, i.e., 23, were subjected to TGS, of which 16 isolates produced acceptable results.

### 3.1. pncA mutations by WGS and TGS

Of 91 isolates whose *pncA* gene sequence was obtained, 67 (74%) exhibited a *pncA* gene mutation, while 24 (26%) were determined to be 'wild-type' by comparison to the H37Rv reference sequence (Table 1). Of the 67 *pncA* gene mutations, 45 distinct *pncA* gene mutational variations were determined. Four isolates carried double mutations, and the remaining 63 isolates harbored a single mutation. Mutations were diverse, i.e., amino acid substitution, stop-translation, deletion, insertion, and nucleotide frameshifts at various positions throughout the coding or promoter regions. Seven mixed-strain substitution mutations were observed, and 2 isolates harbored a deletion mutation in the promoter region (Table 1). A total of 11 novel *pncA* gene mutations, designated 'unknown', i.e., not previously described in the literature, are reported here (Table 1). Novel mutational types included amino acid substitutions ($N = 5$), nucleotide frameshifts ($N = 4$), a 2–amino acid deletion ($N = 1$), and a nucleotide promoter deletion ($N = 1$). Of 16 isolates with novel *pncA* mutations, 6 were resistance conferring, 2 were susceptible, and 8 were not tested using Bactec MGIT 960 (Table 1). Phenotypic PZA susceptibility was noted in 5 of 55 (9%) isolates containing a *pncA* gene mutation that was previously shown to be resistance conferring. However, 4 of these 5 (80%) exhibiting DST discordancy were shown to be heterogenous, harboring both resistant and susceptible mutations (Table 1).

**Table 1**
Summary of *pncA* gene mutation types in 91 Ukraine *M. tuberculosis* clinical isolates characterized by whole genome and targeted gene sequencing.

| No. of isolates | | | Sequencing result by: | Pyrazinamide result by: | |
|---|---|---|---|---|---|
| | Mutation/position | Mutation type | WGS/TGS (no. of isolates) | Genomic analysis[a] (ref no.) | Bactec MGIT 960 |
| 3 | Promoter A(−11)G | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | Promoter ΔG(−5) | Deletion of g | WGS | Unknown | N/A |
| 1 | M1T/M, I31S/I | Substitutions (mixed) | WGS | Resistant (Kyung et al., 2001), Resistant (Mphahlele et al., 2008) | Sensitive |
| 1 | A3P | Substitution | TGS | Resistant (Kyung et al., 2001) | N/A |
| 1 | A3P/A | Substitution (mixed) | WGS | Resistant (Kyung et al., 2001) | Sensitive |
| 1 | 4 frameshift (cgTTG) | Frameshift insertion cg | WGS | Unknown | N/A |
| 1 | L4W, ΔIV (6,7) | Substitution, deletion of I and V | WGS | Resistant (Kyung et al., 2001), Unknown | Resistant |
| 3 | ΔIV (6,7) | Deletion of I and V | WGS | Unknown | Resistant |
| 1 | Q10H | Substitution | WGS | Unknown | Resistant |
| 1 | Q10P | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | Q10R, V128G/V | Substitution, substitution (mixed) | WGS | Resistant (Kyung et al., 2001), Resistant (Kyung et al., 2001) | Sensitive |
| 1 | D12A | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | 16Frameshift(GGgT) | Frameshift insertion g | WGS | Unknown | N/A |
| 2 | G17D | Substitution | WGS *(1)*, TGS *(1)* | Resistant (Kyung et al., 2001) | Resistant |
| 2 | I31S | Substitution | WGS | Resistant (Mphahlele et al., 2008) | N/A |
| 1 | D49N, V130 L | Substitutions | WGS | Resistant (Mphahlele et al., 2008), Resistant (Mphahlele et al., 2008) | N/A |
| 2 | F58 L | Substitution | WGS *(1)*, TGS *(1)* | Resistant (Kyung et al., 2001) | N/A |
| 2 | W68G | Substitution | WGS *(1)*, TGS *(1)* | Resistant (Kyung et al., 2001) | Resistant |
| 1 | W68G/D | Mixed substitution | TGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | W68STOP | termination codon | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | P69L | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | F81S | Substitution | WGS | Resistant (Mphahlele et al., 2008) | Resistant |
| 1 | V93 M | Substitution | WGS | Unknown | Resistant |
| 1 | G97S | Substitution | WGS | Resistant (Kyung et al., 2001) | N/A |
| 1 | A102V/A | Substitution (mixed) | WGS | Resistant (Kyung et al., 2001) | Sensitive |
| 3 | Y103H | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 2 | L120R | Substitution | WGS | Resistant (Miotto et al., 2014) | N/A |
| 1 | 122Frameshift(cggCAA) | Insertion of Glycine (cgg) | WGS | Unknown | Sensitive |
| 1 | V125G | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | 131frameshift (GgTC) | Frameshift insertion g | WGS | Resistant (Barco et al., 2006) | Resistant |
| 3 | 131Frameshift (GggTC) | Frameshift insertion gg | TGS | Resistant (Wade et al., 2004) | Resistant |
| 1 | 132frameshift (cGGT) | Frameshift insertion c | WGS | Unknown | N/A |
| 1 | G132D | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | G132R | Substitution | WGS | Unknown | Sensitive |
| 1 | A134V | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | V139G | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 2 | V139A | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 1 | A146P | Substitution | WGS | Unknown | N/A |
| 1 | L151L/S | Mixed substitution | TGS | Resistant (Mphahlele et al., 2008) | Resistant |
| 1 | V155A | Substitution | WGS | Resistant (Kyung et al., 2001) | Sensitive |
| 2 | T160A | Substitution | WGS | Resistant (Mphahlele et al., 2008) | Resistant |
| 1 | G162A | Substitution | WGS | Resistant (Mphahlele et al., 2008) | N/A |
| 1 | S164P | Substitution | TGS | Resistant (Martin et al., 2006) | N/A |
| 2 | L172P | Substitution | WGS | Resistant (Kyung et al., 2001) | Resistant |
| 4 | T177P | Substitution | WGS *(3)*, TGS *(1)* | Unknown | N/A |
| 1 | T177 T/P | Mixed substitution | TGS | Unknown | Sensitive |
| 1 | V180G | Substitution | WGS | Resistant (Mphahlele et al., 2008) | N/A |
| 1 | V180F | Substitution | WGS | Resistant (Kyung et al., 2001) | N/A |
| 24 | Wild type[b] | None | WGS *(20)*, TGS *(4)* | Sensitive | Sensitive |

N/A = not available; DST using MGIT 960 was not performed.
[a] Unknown = pyrazinamide-conferring mutations not described in the TB Drug Resistance Mutation Database (Kyung et al., 2001) or as referenced (Barco et al., 2006; Martin et al., 2006; Miotto et al., 2014; Mphahlele et al., 2008; Wade et al., 2004).
[b] Wild type as determined by comparison to *M. tuberculosis* H37Rv reference strain.

### 3.2. PZA resistance in MDR/XDR strains

As shown in Table 2, *pncA* gene mutations were detected in 55 of 75 (73%) total isolates analyzed by WGS. Statistical significance for isolates harboring a *pncA* gene mutation in MDR and XDR patients was noted. In MDR patients, *pncA* gene mutations were observed in 31 of 40 (78%) isolates [$P < 0.01$; odd ratio (OR) = 28; 95% confidence interval (CI) = 3–251]. Similarly, 23 of 26 (88%) XDR patients harbored a *pncA* gene mutation ($P < 0.01$; OR = 61; 95% CI = 6–677). In contrast, a *pncA* mutation was noted in only 1 of 9 (11%) drug-sensitive or -resistant isolates, i.e., not MDR or XDR but resistant to at least 1 antibiotic excluding PZA (Table 2).

## 4. Discussion

TB is a disease of global importance that requires treatment with a combination of antimicrobial drugs that include PZA in both short- and long-term MDR/XDR-TB treatment regimens. However, PZA resistance is increasing globally, and accurate diagnosis using phenotype methodologies is problematic due to a variety of issues, the most important being standardization of results (Daum et al., 2014; Kurbatova et al., 2013). Importantly, it has been shown that patients with demonstrated RIF and PZA resistance where first-line treatment includes PZA have a high propensity to develop pre–XDR-TB when PZA is included in the second-line treatment regimen (Fofana et al., 2017). Thus, use of PZA to treat patients that harbor PZA resistance may lead to development of pre-XDR and XDR-TB. Therefore, worldwide detection of *pncA* resistance mutations is critically important especially in RIF-resistant patients that could benefit from a PZA treatment regimen (Bellan et al., 2014; Ramirez-busby and Faramarz, 2015). However, this requires use of rapid, molecular-based PZA resistance detection which currently is not universally available. Turnaround time for *pncA* TGS reported here was approximately 24 h, excluding the time requirement for bioinformatic analysis, as compared to 4 days using WGS methodology. Furthermore, TGS can be utilized in lieu of WGS with primary sputum specimens containing low concentrations of MTB target genomic material and with problematic isolates that contain co-contaminating microbes.

Using WGS and TGS, we detected *pncA* mutations in 67 of 91 (74%) clinical isolates consisting of substitutions, insertions, and deletions along the coding and upstream promoter region (Table 1). Many of these mutations have been previously observed and verified as PZA resistant and indexed in the 'TBDream database' (Sandgren et al., 2009) or elsewhere (Barco et al., 2006; Kyung et al., 2001; Martin et al., 2006; Miotto et al., 2014; Mphahlele et al., 2008; Wade et al., 2004). However, 11 novel *pncA* mutation types previously not reported in the literature were identified. Of these 11 mutation types, DST confirmed 4 were resistant, and 2 were susceptible. The remaining 5 were not analyzed by DST. Clinical isolates harboring *pncA* mutations that were confirmed by DST included 1) an isoleucine and valine deletion (ΔIV(6,7), and 2) and substitution mutations at positions 10, 93, and 177. Additionally, 7 isolates were determined to be mixed-strain samples. It will be critically important to assess by phenotypic DST the 5 clinical isolates harboring novel *pncA* gene mutations to determine whether they do indeed confer PZA resistance. A thorough understanding of *pncA* mutations confirmed as resistance conferring by DST is crucial for guiding molecular diagnostic tests, appropriate drug therapy, and patient care. However, widespread diversity of *pncA* mutations hampers the use of rapid qPCR-based allelic discrimination assays or other single nucleotide polymorphism molecular tests. At present, WGS and TGS low-cost arrays are the most suitable approach for molecular-based *pncA* resistance tests. Miotta et al. (2014) have compiled stratified confidence ordering data of resistance mutations correlating phenotypic results, as well as *pncA* mutations not associated with PZA resistance (Miotto et al., 2014). In this study, several Ukraine isolates contained resistance mutations previously confirmed through phenotypic analysis (*cf.,* Table 1 and Barco et al., 2006; Kyung et al., 2001; Martin et al., 2006; Miotto et al., 2014; Mphahlele et al., 2008; Sandgren et al., 2009; Wade et al., 2004).

A major advantage of NGS over traditional Sanger Sequencing is the depth of coverage, i.e., deep sequencing, and detection of heterogenous nucleotides giving rise to mixed-strain amino acid populations. Detection of wild-type and resistance-conferring mutations in a single isolate can lead to discordancy between previously determined phenotypic and genotypic results particularly when resistance is less than 20% of the total population (Folkvardsen et al., 2013; Zetola et al., 2014). Although the clinical relevance of mixed-strains is unknown, NGS enables analysis at several hundred-fold coverage depth for each loci, which could be important for implementation of an effective PZA drug therapy. For example, a patient harboring a low concentration of resistant organisms to PZA may initially be reported as sensitive by phenotypic testing or Sanger sequencing, but during drug therapy, the PZA-resistant subpopulation may proportionally gain dominance. In this study, alternations in 1 locus, i.e., mixed strains, were observed in the *pncA* gene of 7 clinical isolates (Table 1), which may aid in resolving discordant finds using other tests.

As shown in this study, PZA resistance burden was observed in patients with RIF resistance (Table 2). Fofana et al. (2017) contend that "routine rapid testing for PZA resistance among patients harboring mycobacterial bacilli with demonstrated RIF resistance would be an important means of preventing the emergence of pre-XDR TB." However, currently, the only FDA-approved molecular method for MTB detection is Cepheid's Xpert® MTB/RIF which only detects MTB and RIF resistance. Additionally, it has been observed that as many as 13% of all TB clinical isolates are PZA resistant yet harbor a 'wild-type' *pncA* gene (Simons et al., 2013; Zhang et al., 2013; Zhang and Mitchison, 2003). The basis for resistance in these strains may arise from other genes such as *rpsA* and *panD* encoding a ribosomal protein S1 (*rpsA*, Rv1630) and aspartate alpha-decarboxylase, respectively (Simons et al., 2013; Zhang et al., 2013). Mutations in these genes have been reported in PZA-resistant strains containing 'wild-type' *pncA* and constitute an additional mechanism for PZA resistance (Simons et al., 2013; Zhang et al., 2013). All 75 clinical isolates deduced by WGS reported here were shown to be *panD* and *rpsA* 'wild type' (data not shown). TGS can be expanded beyond the *pncA* gene to include additional MDR-conferring resistance genes, e.g., *rpoB, gyrA/B,* and *katG*. Furthermore, TGS can be used with up to 96 samples/run and carried out using newer low-density sequencing cartridges that will markedly reduce cost per specimen enabling 1) genetic characterization from original sputum specimens, 2) greater coverage depth, 3) fewer false-positive PZA resistant cases, and 4) higher throughput of samples tested.

In this study, discordancy was observed between genotype and phenotype in some isolates (Table 1). In PZA DST, false-positive/negative PZA resistance has been previously noted and attributed to factors

**Table 2**
Prevalence of *pncA* gene mutations in MDR and XDR[a] clinical isolates by whole genome sequencing ($N = 75$).

| | *pncA* gene | | | |
|---|---|---|---|---|
| Resistance level[b] | Mutation | Wild type[d] | *P* value | OR (95% CI) |
| MDR ($n = 40$, 53%) | 31 | 9 | <0.01 | 28 (3–251) |
| XDR ($n = 26$, 35%) | 23 | 3 | <0.01 | 61 (6–677) |
| Resistant[c] ($n = 3$, 4%) | 0 | 3 | - | 1.0 (Ref.) |
| Sensitive[d] ($n = 6$, 8%) | 1 | 5 | - | 1.0 (Ref.) |

[a] Mutation conferring antibiotic resistance in the most prevalent MDR/XDR genes: *rpoB* (rifampin), *katG* and *inhA* (isoniazid), *gyrA/B* (fluoroquinolone), and *rrs/eis* (aminoglycosides). WGS ($N = 75$) was carried out as described under 'Materials and methods.' All mutational comparisons were made to the *M. tuberculosis* H37Rv reference strain.

[b] MDR = multidrug resistance; XDR = extensive-drug resistance; Sensitive = sensitive to first- and second-line antibiotics.

[c] Not MDR but resistant to at least 1 antibiotic drug used for MDR/XDR-TB treatment not including PZA.

[d] According to homology to H37Rv reference strain.

such as growth pH and critical concentration values inherent to DST phenotype assays (Chedore et al., 2010b; Daum et al., 2014; Kurbatova et al., 2013; Piersimoni et al., 2013). However, as reported here, NGS was used to characterize several novel *pncA* gene mutations not previously reported as resistance-conferring mutations (Barco et al., 2006; Kyung et al., 2001; Martin et al., 2006; Miotto et al., 2014; Mphahlele et al., 2008; Sandgren et al., 2009; Wade et al., 2004). One limitation of this study was the lack of corresponding phenotypic DST data on some isolates harboring a novel *pncA* mutation. In clinical isolates where a *pncA* mutation was detected but not phenotypically verified, the nature of the mutation, i.e., conferring of PZA resistance, will require confirmatory phenotypic analysis.

In conclusion, this is the first study using NGS analysis of clinical isolates from the Ukraine to characterize resistance mutations in the *pncA* gene. Several Ukraine isolates harbored mutations not observed in other high-burden TB countries, underscoring the importance of monitoring PZA resistance from geographically diverse populations. Most notably, detection of novel *pncA* gene mutations confirmed by DST provides a broader understanding of *pncA* resistance patterns from this area of the world. The use of routine, high-throughput NGS for detection of PZA resistance may be helpful in guiding effective antibiotic treatment in patients with drug-resistant TB from the Ukraine and other high-prevalence areas of the world.

## Conflict of interest

There is no conflict of interest.

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Luke T. Daum, PhD, is the Chief Scientific Officer at Longhorn Vaccines and Diagnostics.

His research interests include molecular epidemiology and development of diagnostics for detecting MTB, influenza viruses, and other emerging pathogens.

## References

Allana S, Shashkina E, Mathema B, Bablishvili N, Tukvadze N, Shah NS, et al. pncA gene mutations associated with pyrazinamide resistance in drug-resistant tuberculosis, South Africa and Georgia. Emerg Infect Dis 2017;23(3):491–5.

Barco P, Cardoso RF, Hirata RDC, Leite CQF, Pandolfi JR, Sao DN, et al. pncA mutations in the pyrazinamide-resistant Mycobacterium tuberculosis clinical isolates from the southeast region of Brazil. J Antimicrob Chemother 2006;58(5):930–5.

Bellan SE, Pulliam JRC, Dushoff J, Myers LA. Conflict and drug-resistant tuberculosis in the Ukraine. Lancet 2014;384:1500–1.

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 2010a;8(1):300–1.

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 2010b;48(1):300–1.

Daum LT, Fourie PB, Bhattacharyya S, Ismail NA, Gradus S, Maningi NE, et al. Next-generation sequencing for identifying pyrazinamide resistance in Mycobacterium tuberculosis. Clin Infect Dis 2014;58(6):903–4.

Falzon D, Schünemann HJ, Harausz E, González-Angulo L, Lienhardt C, Jaramillo E, et al. World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update. Eur Respir J 2017;22(3):49.

Fofana MO, Shrestha S, Knight GM, Cohen T, White RG, Cobelens F, et al. Multistrain mathematical model to investigate the role of pyrazinamide in the emergence of extensively drug-resistant tuberculosis. Antimicrob Agents Chemother 2017;61(3):1–12.

Folkvardsen DB, Thomsen VO, Rigouts L, Rasmussen EM, Bang D, Bernaerts G, et al. Rifampin heteroresistance in Mycobacterium tuberculosis cultures as detected by phenotypic and genotypic drug susceptibility test methods. J Clin Microbiol 2013;51:4220–2.

Global TB Programme. Updated interim critical concentrations for first-line and second-line DST (as of May 2012). WHO guidelines for the programmatic management of drug-resistant tuberculosis. Available at: http://www.stoptb.org/wg/gli/assets/documents/Updated%20critical%20concentration%20table_1st%20and%202nd%20line%20drugs.pdf, 2012.

Huy NQ, Lucie C, Hoa TTT, Hung NV, Lan NTN, Son NT, et al. Molecular analysis of pyrazinamide resistance in Mycobacterium tuberculosis in Vietnam highlights the high rate of pyrazinamide resistance-associated mutations in clinical isolates. Emerg Microbes Infect 2017;6(10).

Kurbatova EV, Cavanaugh JS, Dalton T, Click ES, Cegielski JP. Epidemiology of pyrazinamide-resistant tuberculosis in the United States, 1999–2009. Clin Infect Dis 2013;57(8):1081–93.

Kyung LW, Lee JM, Jung KS. Characterization of pncA mutations of pyrazinamide-resistant Mycobacterium tuberculosis in Korea. J Korean Med Sci 2001;16:537–43.

Liu W, Chen J, Shen Y, Jin J, Wu J, Sun F, et al. Phenotypic and genotypic characterization of pyrazinamide resistance among multidrug-resistant Mycobacterium tuberculosis clinical isolates in Hangzhou, China. Clin Microbiol Infect 2017;59(3):1690–5.

Martin A, Takiff H, Vandamme P, Swings J, Palomino JC, Portaels F. a new rapid and simple colorimetric method to detect pyrazinamide resistance in Mycobacterium tuberculosis using nicotinamide. J Antimicrob Chemother 2006;58(2):327–31.

Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, et al. Mycobacterium tuberculosis pyrazinamide resistance determinants: a multicenter study. MBio 2014;5(5). 01819-14.

Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsaker T, Muthivhi T, et al. Pyrazinamide resistance among South African multidrug-resistant Mycobacterium tuberculosis isolates. J Clin Microbiol 2008;46:3459–64.

Pang Y, Zhu D, Zheng H, Shen J, Hu Y, Liu J, et al. Prevalence and molecular characterization of pyrazinamide resistance among multidrug-resistant Mycobacterium tuberculosis isolates from southern China. BMC Infect Dis 2017;17(1):711.

Pavlenko E, Barbova A, Hovhannesyan A, Tsenilova Z, Slavuckij A, Shcherbak-Verlan B, et al. Alarming levels of multidrug-resistant tuberculosis in Ukraine: results from the first national survey. Int J Tuberc Lung Dis 2018;22(2):197–205.

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;51(1):291–4.

Ramirez-busby SM, Faramarz Valafar. Systematic review of mutations in pyrazinamidase associated with pyrazinamide resistance in mycobacterium tuberculosis clinical isolates. Antimicrob Agents Chemother 2015;59(9):5267–77.

Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med 2009;6(2):e2. TBDream Website available at: https://tbdreamdb.ki.se/Data/DrugArea.aspx?AreaID=PZA.

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;51(1):382.

Wade MM, Volokhov D, Peredelchuk M, Chizhikov V, Zhang Y. Accurate mapping of mutations of pyrazinamide-resistant Mycobacterium tuberculosis strains with a scanning-frame oligonucleotide microarray. Diagn Microbiol Infect Dis 2004;49(2):89–97.

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).

World Health Organization (WHO). Policy guidance on drug-susceptibility testing (DST) of second-line antituberculosis drugs. Report WHO/HTM/TB/2008.392. Geneva, Switzerland: World Health Organization; 2008. Available at: http://apps.who.int/iris/bitstream/10665/70500/1/WHO_HTM_TB_2008.392_eng.pdf.

World Health Organization (WHO). treatment guidelines for drug-resistant tuberculosis, 2016 update. Geneva: World Health Organization, 2016a. Available at: https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0093618/.

World Health Organization (WHO). Ukraine — tuberculosis profile. Web 24 Mar. 2017 World Health Organization; 2016b. Available at: https://extranet.who.int/sree/Reports?op=Replet&name=%2FWHO_HQ_Reports%2FG2%2FPROD%2FEXT2FTBCountryProfile&ISO2=UA&LAN=EN&outtype=html.

Zetola NM, Shin SS, Tumedi KA, Moet K, Ncube R, Nicol M, et al. Mixed Mycobacterium tuberculosis complex infections and false-negative results for rifampin resistance by GeneXpert MTB/RIF are associated with poor clinical outcomes. J Clin Microbiol 2014;52:2422–9.

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;2(6), e34.

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

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