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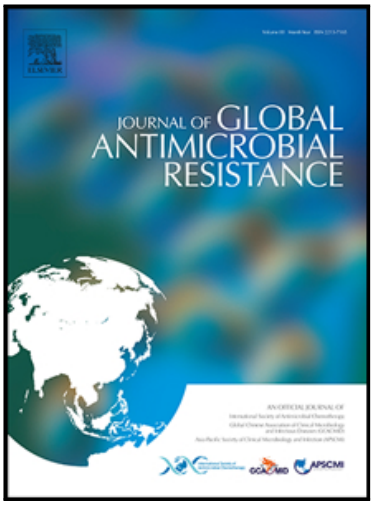

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Original article

Next-generation sequencing to characterize pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from two Balkan countries

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Highlights

- Comparison of NGS to conventional BACTEC MGIT 960 revealed very strong agreement.
- The majority of PZA-resistant TB isolates harboured mutations in *pncA*.
- The in-frame *pncA* mutations were randomly distributed along the entire *pncA* gene.
- A-11G *pncA* promoter mutation was the most prevalent genetic alteration.

Abstract:

Objectives: Next-generation sequencing (NGS) provide a comprehensive analysis of the genetic alterations that are most commonly linked with pyrazinamide (PZA) resistance. There are no studies reporting molecular background of PZA resistance in TB isolates from Balkan Peninsula. We aimed to examine the feasibility of full-length analysis of a gene linked with PZA resistance, *pncA*, using Ion Torrent technology in comparison to phenotypic BACTEC MGIT 960 DST in clinical TB isolates from two countries of the Balkan Peninsula.

Methods: Between 1996 and 2017, we retrospectively selected 61 TB isolates. To identify gene variants related to drug resistance in genomic DNA extracted from TB isolates, AmpliSeq libraries were generated automatically using the AmpliSeq™ Kit for Chef DL8 and the Ion AmpliSeq TB Research Panel.

Results: Of all 61 TB isolates included, 56 TB were phenotypically resistant to any antibiotic. Among them, 38/56 (67.9%) TB isolates were phenotypically resistant to pyrazinamide and *pncA* mutations were detected in 33/38 cases (86.8%). A mutation in the *pncA* promoter region was the most prevalent genetic alteration, detected in eight TB isolates. Comparison of NGS to conventional BACTEC MGIT 960 DST revealed very strong agreement (90.2%) between the two methods in identifying PZA resistance, with high sensitivity (89.5%) and specificity (95.7%) for NGS.

Conclusions: Detection of PZA resistance using NGS seems to be a valuable tool for surveillance of TB drug resistance also in the Balkan Peninsula, with great potential to provide useful information at least one weak earlier than is possible with phenotypic DST.

Key words: Tuberculosis, *Mycobacterium tuberculosis*, next-generation sequencing, *pncA*, pyrazinamide, phenotypic drug susceptibility testing

1. Introduction:

Slovenia and North Macedonia are two small countries of former Yugoslavia, each with cca. 2 million inhabitants and with a low incidence of tuberculosis; their incidence rates in 2019 were 5.4 and 12.0, respectively. In both countries, the percentage of drug-resistant cases is low, with sporadic cases of multidrug resistant tuberculosis (MDR-TB) [1]. In Slovenia, in the years between 1996 and 2018, the average rate of any drug resistance against first-line antibiotics (including streptomycin) was 4.1%. The incidence of drug resistance has been gradually declining over the years and accounted for 3.3% of cases in 2018 [2,3]. For North Macedonia, the average rate of any drug resistance accounted for 10.8% of cases (drug susceptibility testing (DST) for pyrazinamide (PZA) was not performed at that time) and dropped to 5.8% of cases in 2017 [3]. On the other side, knowledge on molecular background of drug resistance in TB isolates from both countries is very limited [3].

Despite major efforts to control the global TB burden, the emergence of drug resistance remains a great concern. Therefore, rapid and accurate diagnosis of drug resistance is essential for timely initiation of appropriate and optimal TB treatment [4,5].

PZA is an important first-line drug that acts synergistically with rifampicin (RIF) in the treatment of tuberculosis. It has a unique ability to kill semidormant bacilli that persist in the acidic environments inside macrophages. When added

to treatment schemes containing rifampicin (RIF) and isoniazid (INH), it is able to kill persisting TB bacilli, allowing treatment to be shortened from 9-12 months to 6 months [6,7].

The mechanisms of action and resistance to PZA in TB are not completely understood. PZA is a prodrug activated in the cytoplasm by mycobacterial nicotinamidase/pyrazinamidase (*PZase*) into the active form, pyrazinoic acid (POA) which through mechanism descried elsewhere disrupts membrane permeability and transport, leading to cellular damage [8,9].

PZase is encoded by the 561-nucleotide *pncA* gene. Studies have strongly correlated mutations in the *pncA* gene and its promoter region with PZA resistance in TB [10-13]. Genetic alterations in other genes (*rpsA, panD, clpC1*) have been described to be responsible for PZA resistance in a smaller percentage of TB isolates [7,14-16].

Despite the widespread use of PZA in TB treatment regimens, the World Health Organization (WHO) does not include PZA in the group of antimycobacterial drugs for which resistance is routinely tested due to the lack of reliable culture-based susceptibility testing; the existing tests have poor reproducibility and produce false-positive results for PZA resistance [4]. Several factors contribute to the inaccuracy and reduced reproducibility of PZA susceptibility testing e.g. the size of the mycobacterial inoculum used, inability of some clinical TB isolates to grow in acidic environment [14,17-21]. Another shortcoming of

classical phenotypic DST is that it produces a binary result of susceptibility or resistance rather than the exact minimal inhibitory concentration (MIC) of the drug for the isolate. Therefore, PZA has often been excluded from efforts to develop methods to identify drug resistance. Since PZA resistance testing is not routinely performed in all clinical TB laboratories, the prevalence of PZA resistance is not well known. A relatively high proportion of PZA-resistant TB strains among MDR-TB isolates has been reported [22,23]. Concordant with these observations, Chang et al [18] reported in their systematic review that the median prevalence of pyrazinamide resistance was 51% (range: 31% to 89%) in MDR TB isolates and 5% (range: 0% to 9%) in non-MDR TB isolates.

Taking in the account all the limitations of classical phenotypic DST, molecular methods hold great promise in detection of PZA resistance. In January 2021, WHO reported high diagnostic accuracy of high complexity hybridization-based nucleic acid amplification tests (NAATs; e.g. Genoscholar PZA-TB II, Nipro) for the detection of PZA resistance in MTB isolated from patients with bacteriologically confirmed pulmonary TB (24).

Furthermore, the reference method for PZA susceptibility testing is sequencing, with specific mutations interpreted as predictors of drug-resistant TB phenotypes [4,25]. It has been reported that NGS can accurately predict clinically relevant resistance in TB isolates, even in those with lower MICs where phenotypic DST is unreliable [4,16,25-27]. Recent studies [25,27]

suggested a standardized and comprehensive approach for the interpretation of resistance-associated mutations for PZA; they proposed five groups of *pncA* mutations to inform the use of PZA in TB treatment schemes and the role of additional DST for PZA. Furthermore, NGS, especially targeted approaches, enables the rapid identification of TB and determination of resistance against key antibiotics. Furthermore, one of the main advantages of targeted NGS when compared to WGS is the possibility to determine resistance directly in primary samples, without the need of performing culture, thus significantly shortening the turn-around-times [28].

In the present study, we examined the feasibility of a full-length gene analysis for the PZA resistance-related gene *pncA* using Ion Torrent technology and compared the results with those obtained from conventional phenotypic BACTEC MGIT 960 DST in 61 clinical TB isolates from two Balkan countries, Slovenia and North Macedonia.

Materials and methods:

1.1. Selection of TB isolates: We retrospectively selected 33 drug resistant TB isolates that were collected between 1996 and 2017 from various clinical samples of Slovenian patients and subjected to phenotypic DST testing according to routine procedures in the Laboratory for Mycobacteria (University Clinic Golnik, Slovenia). Between 1999 and 2010, the National Laboratory for Mycobacteria (Institute for

Pulmonary Diseases and Tuberculosis Skopje, North Macedonia) sent 23 TB isolates to our laboratory for assistance with phenotypic DST. All TB isolates included in this study were retrieved from the Slovenian National Mycobacterial Strain Collection. The TB isolates included were either mono-, poly- or MDR resistant. For control purposes, we also randomly selected five TB isolates known to be susceptible to first-line anti-TB drugs. In total, 61 TB isolates were include in our study.

1.2. Phenotypic drug susceptibility (DST) testing: Phenotypic drug resistance to PZA was determined using the BACTEC MGIT 960 System (BD Diagnostic System, NJ, USA) from pure cultures of TB isolates. The critical concentration (CC) of PZA was 100 µl/ml. In the case of discrepant results between NGS analysis and phenotypic BACTEC MGIT 960 DST, the phenotypic DST was repeated under the same conditions.

1.3. DNA extraction: Mycobacterial genomic DNA was isolated from pure cultures of TB isolates using a previously described protocol [29]. Purified genomic DNA was stored at -20 °C in the Slovenian National Mycobacterial DNA Collection until further processing.

1.4. AmpliSeq Library preparation, sequencing, data analysis and interpretation: Nucleic acid quality and quantity were assessed using a NanoDrop 2000 (Thermo Scientific, MA, USA) followed by agarose

gel electrophoresis. All DNA samples were normalized to 10 ng in 15 µl of starting sample dilution. To identify gene variants related to drug resistance in genomic DNA extracted from MTB isolates, AmpliSeq libraries were generated using the AmpliSeq™ Kit for Chef DL8 and the Ion AmpliSeq TB Research Panel in the Ion Chef system. This panel amplifies 109 amplicons in two highly multiplexed PCRs covering the coding sequences of eight genes related to drug resistance (*pncA* and other genes related to TB drug resistance*: embB*, *eis*, *gyrA*, *inhA*, *katG*, *rpoB*, and *rpsL*). NGS libraries were prepared automatically using the Ion Chef instrument. The automated protocol performs targeted amplification, digestion, ligation, and normalization on eight samples without any user intervention. The prepared libraries were then automatically clonally amplified, enriched and sequenced on two Ion 530 Chips using the Ion Chef and Ion S5 instruments. Signal processing, base calling and variant calling analysis were performed with Torrent Suite software version 5.6 (all reagents, instruments and software from Thermo Fisher Scientific, MA, USA). The sequencing data were analyzed manually, comparing the determined variants with published data, data available in database provided by Köser et al [27] and data available in the web tool PhyResSE [30]. The sequence of MTB H37Rv (NC_000962.3) was used as the reference sequence. The resistance genotyping profiles obtained with the manual approach were

compared to the results of phenotypic DST testing. Sequence data are available in the SRA NCBI database under BioProject accession number PRJNA551916. The mean coverage depth obtained by NGS for the *pncA* gene was 2361x. Threshold used to call for variants was set at 10%.

1.5. *Statistics*: Sensitivity and specificity for NGS were calculated using GraphPad Prism v 6.04 (GraphPad Software, Inc., CA, USA).

2. Results

2.1. *Phenotypic DST testing:* Of all 61 TB isolates included, 56 TB were phenotypically resistant to any antibiotic. After repeated BACTEC MGIT 960 DST, 38 (38/56, 67.9%) isolates showed a PZA-resistant phenotype. Of those 38 TB isolates, six (6/38, 15.8%) isolates were pyrazinamide monoresistant, and the remaining 32 (32/38, 84.2%) isolates were resistant to other antibiotics (MDR: 25/38, 65.8%; polyresistant: 7/38, 18.4%). The data on the phenotypic resistance profiles of the TB isolates included in this study and information on the presence of genetic variations in the *pncA* gene are presented in Table 1.

2.2. *NGS analysis*: Among the 38 TB isolates that were phenotypically resistant to pyrazinamide, mutations in the *pncA* gene were detected in 33 cases (33/38; 86.8%); no *pncA* mutations were noted in the remaining five (5/38; 13.2%) PZA-resistant TB isolates. A mutation in

the *pncA* gene (specifically, Pro69Leu) linked with PZA resistance was also detected in one TB isolate (1/23; 4.3%) that was phenotypically sensitive to PZA. When the BACTEC MGIT 960 DST (CC of PZA was 100 µl/ml) repeated, that particular TB isolate remained sensitive to PZA. No *pncA* mutations were detected in the remaining 22 (22/23; 95.7%) PZA-susceptible TB isolates. The in-frame *pncA* mutations detected in our study were randomly distributed along the entire *pncA* gene. In total, we detected 18 different *pncA* mutations using Ion Torrent technology. The most prevalent genetic alteration was a mutation at position –11 in the *pncA* promoter region, which was detected in eight PZA-resistant TB isolates. Two other mutations were also detected at some frequency: Lys96Gln, which was detected in five PZA-resistant TB isolates, and Val(s)155Gly, which was detected in four PZA-resistant TB isolates. Frameshifts due to nucleotide insertions or deletions were detected in four TB isolates. Other mutations were detected in only one or two TB isolates (Table 1). Overall, Ion Torrent NGS technology was successfully used to predict the PZA resistance profile, leading to a high rate of agreement between both methods and the corresponding good analytical sensitivity and specificity of NGS analysis (Table 2).

3. Discussion

Rapid and accurate detection of TB drug resistance is important for successful disease treatment and in prevention of TB dissemination. PZA is crucial drug due to its ability to kill semidormant bacilli, which allows significant shortening of TB treatment. Culture-based DST still lacks reproducibility and results in false-positive PZA resistance. Many clinical laboratories, even in Europe, still do not perform phenotypic DST for PZA.

To our knowledge, data describing the comprehensive comparison of NGS and phenotypic DST for determining any kind of drug resistance in TB from countries of former Yugoslavia are very limited [31]. Furthermore, there are no studies reporting which *pncA* mutations are linked with PZA resistance in TB isolates in Slovenia and North Macedonia; to our knowledge, this is the first study reporting such a comparison for determining the PZA resistance of TB isolates in the Balkan region. In our study, the comparison of NGS analysis to conventional BACTEC MGIT 960 DST revealed a high degree of concordance (90.2%) between the two methods in identifying PZA resistance, with a high sensitivity (89.5%) and specificity (95.7%) for NGS analysis. These findings are in concordance with the results of a systemic review [18], which reported a high median sensitivity of 92% (range: 87–95%) and a high median specificity of 93% (range: 88-97%) for PCR-DNA-based sequencing. Furthermore, a large study conducted in the frame of the CRyPTIC Consortium [31], with over 10,000 TB isolates, reported that PZA resistance was correctly predicted with

91.3% sensitivity and PZA susceptibility was correctly predicted with 96.8% specificity with whole-genome sequencing (WGS) compared to phenotypic DST.

The majority of PZA-resistant TB isolates (33/38; 86.8%) included in our study harboured mutations/genetic alterations in the *pncA* gene. Mutations were randomly distributed along the entire length of the *pncA* gene. This observation is concordant with other published reports that found *pncA* mutations in 70% to 96% of PZA-resistant strains [16,21,32-37].

In five TB isolates (5/38; 13.2%) that were phenotypically resistant to PZA, we did not identify mutations in the *pncA* gene. These TB isolates remained PZA resistant after standard phenotypic DST (BACTEC MGIT 960 with PZA CC of 100 µl/ml) was repeated. Similarly, other published reports noted the presence of PZA-resistant strains without *pncA* mutations, the percentage of which ranged from 3 to 30% [7,9,32]. Although mutations in *pncA* have been recognized as the main cause of PZA resistance in TB, recent studies suggest that there seems to be a secondary mechanism of resistance to PZA. This indicates that other genes and mechanisms are involved in PZA resistance. Sheen et al [8] concluded on the basis of their study that the POA efflux rate was the best predictor for PZA resistance. The results of these studies suggest that tests that detect *pncA* mutations or *PZase* activity are likely to be less predictive of real PZA resistance than tests that measure the rate of POA efflux. Concordant with

these observations, some recent studies demonstrated that overexpression of and/or mutations in several PZA/POA binding efflux proteins caused resistance to PZA in some TB strains [9,38]. Moreover, genetic alterations in several other genes involved in energy metabolism and protein degradation have been implicated in PZA resistance, including *rpsA* (coding for ribosomal protein S1), *panD* (aspartate 1-decarboxylase) and *clpC1* (caseinolytic protein) [7-9,14-16].

In the frame of our study, we observed relatively high percentage of isolates phenotypically resistant to PZA among rifampicin susceptible isolates (13/20; 65.0%). Four of the mentioned isolates (SLO-4292, SLO-818, SLO-874 and SLO-1301) all carried missense mutation Val(s)155Gly, which might indicate clonal relation of those particular isolates. All four isolates were isolated from patients who live in the same geographical location (approximately ≤20 km apart). Three isolates (SLO-818, SLO-874 and SLO-1301) shared 100% identical RFLP (restriction fragment length polymorphism) pattern (data not shown). Because over the years we changed genotyping method, the forth isolate (SLO-4292) do not have available RFLP pattern. The use of WGS as a genotyping method would further explore possible epidemiological link between TB cases from which mentioned MTB strains were isolated.

Interesting enough, the only mutation that was detected in both phenotypically resistant and phenotypically sensitive TB isolates was the point mutation Pro69Leu, which was found in one PZA-resistant (MDR TB; MK-3822) and one

PZA-susceptible TB isolate (SLO-4498). This particular *pncA* mutation was previously described in one TB isolate susceptible to PZA and isolated from Chinese patient [23]. According to these results, the Pro69Leu mutation may not be strongly associated with phenotypic PZA resistance. Therefore, further exploration is needed to assess the confidence level that is set for grading mutations associated with phenotypic resistance proposed by Miotto et al [25] and Köser et al [27]. Nevertheless, one should take into consideration that often only one critical concentration is tested in classical phenotypic DST, while the level of resistance varies between TB strains. A proportion of isolates with low-level (borderline) resistance do not grow at tested critical concentration and therefore test as sensitive [16].

Moreover, discordance between phenotypic DST and NGS was the highest in TB isolates with monoresistance to PZA; of total 6 TB isolates with phenotypic resistance only to PZA three of them had no mutation detected in *pncA* gene with NGS. After repeated PZA DTS all three mentioned TB isolates tested PZA resistant. This observation deserves further investigation in larger number of PZA monoresistant TB isolates.

To conclude, the identification of *pncA* mutations with targeted NGS proved to be highly concordant with standard DST in predicting PZA resistance. However, we found some TB isolates that remained PZA resistant even after repeated phenotypical DST and harboured no *pncA* mutations; given the shortcomings of

PZA DST mentioned above we cannot exclude that these are not false positives. On the other hand, one PZA-sensitive TB isolate harboured a *pncA* mutation which is according to the published literature most likely linked with PZA resistance. This indicates that the identification of true drug resistance is a complex process that needs standardization and further research. Nevertheless, the detection of PZA resistance using targeted NGS analysis seems to be a valuable tool for surveillance of drug resistance in TB, with great potential to provide useful information at least one week earlier than phenotypic DST. Even greater value of targeted NGS approach would be to include other genes and gene variants also implicated in PZA resistance (*rpsA, panD, clpC1*).

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Competing Interests

The authors declare no conflicts of interest.

Ethical approval

The study was approved by the Slovenian National Medical Ethics Committee (approval number 0120-94/2021/3).

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Table 1: TB isolates (*N* = 61) according to phenotypic resistance and/or with identified mutations/gene alterations in *pncA* gene

Phenotypic resistance	TB isolate	Phenotypic resistance to other antituberculotics	*pncA* mutations*		Allele frequency (%)
			Mutation site, nucleotide changes	Amino acid changes	
TB isolates resistant to PZA (N = 38)					
MDR (*N* =25)	**MK-1848/04**	INH, RIF, EMB, SM	532 GC insertion	**179frameshift(ccAGC)**	**100.0**
	MK-3823/10	INH, RIF, EMB, FQ, SM	532 GC insertion	**179frameshift(ccAGC)**	**98.6**
	SLO-3128/08	INH, RIF, SM	-11 T>C	**A-11G pncA promoter**	**100.0**
	SLO-423/01	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**100.0**
	SLO-574/01	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**100.0**
	SLO-636/96	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**100.0**
	SLO-828/02	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**99.7**
	MK-3224/08	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**88.5**
	MK-3572/10	INH, RIF, EMB, SM	-11 T>C	**A-11G pncA promoter**	**100.0**
	MK-3648/10	INH, RIF, EMB	-11 T>C	**A-11G pncA promoter**	**100.0**
	MK-2617/06	INH, RIF, EMB, SM	7 G>T	**Missense Ala3Glu**	**99.8**
	MK-1858/04	INH, RIF, EMB, SM	424 G>A	**Missense Thr142Met(s)**	**99.8**
	MK-2603/06	INH, RIF	201 A>G	**Missense Trp68Arg**	**98.7**
	MK-2758/06	INH, RIF, EMB, SM	296 G>T	**Nonsense Tyr99STOP**	**100.0**
	MK-620/99	INH, RIF, EMB, SM	225 T>G	**Missense Thr76Pro**	**100.0**
	MK-2992/07	INH, RIF, EMB, SM	394 C>T	**Missense Gly132Asp**	**100.0**
	SLO-635/96	INH, RIF, EMB	10 A>G	**Missense Leu(s)4Ser**	**99.2**
	SLO-135/95	INH, RIF, EMB, FQ, SM	285 T>C	**Missense Lys96Glu**	**99.5**
	SLO-136/96	INH, RIF, EMB, FQ, SM	285 T>C	**Missense Lys96Glu**	**99.2**
	SLO-137/96	INH, RIF, EMB, SM	285 T>C	**Missense Lys96Glu**	**99.2**
	SLO-140/97	INH, RIF, EMB	285 T>C	**Missense Lys96Glu**	**99.8**
	SLO-1660/03	INH, RIF, EMB, SM	285 T>C	**Missense Lys96Glu**	**100.0**
	MK-3822/10	INH, RIF	205 G>A	**Missense Pro69Leu**	**12.0**
	SLO-3004/07	INH, RIF, EMB, SM	130 A>C	**Missense Val44Gly**	**99.7**
	MK-3571/10	INH, RIF	389 A>G	**Missense Glu130Gly**	**100.0**
Polyresistance (*N* = 7)	**MK-3223/08**	INH, EMB, FQ, SM	390 C insertion	**131frameshift(gGTC)**	**100.0**
	SLO-3241/09	INH, SM	420 G>A	**Nonsense Gln141STOP**	**99.8**
	SLO-4292/16	SM	463 A>C	**Missense Val(s)155Gly**	**99.2**
	SLO-818/01	SM	463 A>C	**Missense Val(s)155Gly**	**100.0**
	SLO-874/02	SM	463 A>C	**Missense Val(s)155Gly**	**100.0**
	SLO-1365/02	INH	211 T>C	**Missense His71Arg**	**100.0**
	SLO-4056/14	INH	No mutation		
Monoresistance	**SLO-4250/15**		158 GTCGAT deletion	**Frameshift**	**100.0**

to PZA (*N* = 6)	**SLO-1301/96**		463 A>C	**Missense Val(s)155Gly**	**100.0**
	SLO-4339/16		33 C>G	**Missense Asp12His**	**99.5**
	SLO-3680/11		No mutation		
	SLO-4262/15		No mutation		
	SLO-631/96		No mutation		

Table 1. ….continued…

Phenotypic resistance	**TB isolate**	**Phenotypic resistance to other antituberculotics**	***pncA* mutations***		**Allele frequency (%)**
			Mutation site, nucleotide changes	**Amino acid changes**	
TB isolates sensitive to PZA (N = 23)					
MDR (*N* = 13)	**SLO-4498/17**	INH, RIF, EMB, SM	205 G>A	**Missense Pro69Leu**	**99.5**
	MK-2862/07	INH, RIF	No mutation		
	SLO-2496/05	INH, RIF, SM	No mutation		
	MK-3567/10	INH, RIF	No mutation		
	MK-2229/05	INH, RIF, EMB, SM	No mutation		
	MK-3566/10	INH, RIF	No mutation		
	MK-2323/05	INH, RIF	No mutation		
	MK-3225/08	INH, RIF	No mutation		
	MK-3113/08	INH, RIF	No mutation		
	MK-2854/07	INH, RIF, EMB	No mutation		
	SLO-811/01	INH, RIF	No mutation		
	MK-3222/08	INH, RIF	No mutation		
	SLO-257/99	INH, RIF, EMB, SM	No mutation		
Monoresistance to INH (*N* = 2)	**SLO-4141/14**	INH	No mutation		
	SLO-4241/15	INH	No mutation		
Monoresistance to RIF (*N* = 3)	**SLO-2391/05**	RIF	No mutation		
	SLO-395/01	RIF	No mutation		
	SLO-633/96	RIF	No mutation		
Sensitive (*N* = 5)	**SLO-4408/17**		No mutation		
	SLO-4407/17		No mutation		
	SLO-4371/16		No mutation		
	SLO-4373/16		No mutation		
	SLO-4372/16		No mutation		

Footnote: MDR: multi-drug resistant; INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol; PZA: Pyrazinamide; SM: Streptomycin; FQ: fluoroquinolones, **pncA* mutations were identified using next-generation sequencing (Ion Torrent, Ion AmpliSeq TB Research Panel, Thermo Fisher Scientific).

Table 2: Comparison of next-generation sequencing (NGS) and phenotypic (BACTEC MGIT 960) pyrazinamide susceptibility testing (DST) in *Mycobacterium tuberculosis* (MTB) isolates (*N* = 61)

NGS	Phenotypic DST			% of agreement
	Resistant	Susceptible	Total	
Pyrazinamide: *pncA*				
mutated	34	1	**35**	**91.8**
non-mutated	4	22	**26**	
Total	**38**	**23**	**61**	
	%			**95% CI**
Sensitivity	**89.5**			(75.2 - 97.1)
Specificity	**95.7**			(78.1 - 99.9)

Footnote: 95% CI: 95% confidence interval; NGS: next-generation sequencing (Ion Torrent, Ion AmpliSeq TB Research Panel, Thermo Fisher Scientific)