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Detection of multidrug-resistance in *Mycobacterium tuberculosis* by phenotype- and molecular-based assays

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Abstract

Background The whole-genome sequencing (WGS) is becoming an increasingly effective tool for rapid and accurate detection of drug resistance in *Mycobacterium tuberculosis* complex (MTBC). This approach, however, has still been poorly evaluated on strains from Central and Eastern European countries. The purpose of this study was to assess the performance of WGS against conventional drug susceptibility testing (DST) for the detection of multi-drug resistant (MDR) phenotypes among MTBC clinical strains from Poland and Lithuania.

Methods The study included 208 MTBC strains (130 MDR; 78 drug susceptible), recovered from as many tuberculosis patients in Lithuania and Poland between 2018 and 2021. Resistance to rifampicin (RIF) and isoniazid (INH) was assessed by Critical Concentration (CC) and Minimum Inhibitory Concentration (MIC) DST as well as molecular-based techniques, including line-probe assay (LPA) and WGS. The analysis of WGS results was performed using bioinformatic pipeline- and software-based tools.

Results The results obtained with the CC DST were more congruent with those by LPA compared to pipeline-based WGS. Software-based tools showed excellent concordance with pipeline-based analysis in prediction of RIF/INH resistance. The RIF-resistant strains demonstrated a relatively homogenous MIC distribution with the mode at the highest tested MIC value. The most frequent RIF-resistance conferring mutation was *rpoB* S450L. The mode MIC for INH was two-fold higher among double *katG* and *inhA* mutants than among single *katG* mutants. The overall rate of discordant results between all methods was calculated at 5.3%. Three strains had discordant results by both genotypic methods (LPA and pipeline-based WGS), one strain by LPA only, three strains by MIC DST, two strains by both MIC DST and pipeline-based WGS, and the remaining two strains showed discordant results with all three methods, compared to CC DST.

Conclusions Considering MIC DST results, current CCs of the first-line anti-TB drugs might be inappropriately high and may need to be revised. Both molecular methods demonstrated 100% specificity, while pipeline-based WGS had slightly lower sensitivity for RIF and INH than LPA, compared to CC DST.

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Keywords MDR-TB, Drug susceptibility testing, Minimum inhibitory concentration, Whole-genome sequencing

Background

Rifampicin-resistant and multi-drug-resistant tuberculosis (RR/MDR-TB) rates in the European Union and European Economic Area (EU/EEA) remain below the World Health Organization (WHO) target goals. Although the incidence of TB is steadily declining in Lithuania, the country still ranks among the leaders in terms of notification rates of new and previously treated RR/MDR-TB cases (3.8 RR/MDR-TB cases per 100,000 vs. 0.2 RR/MDR-TB cases per 100,000 in the EU/EEA in 2021). In contrast, the neighboring Poland has one of the lowest RR/MDR-TB notification rates in the EU/EEA (0.1/100,000 in 2021) [1].

For many years there were only two WHO-recommended molecular methods for RIF/INH resistance prediction, i.e. (i) GeneXpert MTB/RIF (Cepheid, USA) and (ii) LPA (GenoType MTBDRplus (HAIN, Germany). Whereas GeneXpert MTB/RIF is based on the detection of the most frequent RIF resistance-conferring mutations within an 81-bp long fragment of the *rpoB* gene of MTBC strains, LPA detects resistance to RIF/INH through identification of mutations in *rpoB*, *katG*, and *inhA* genes. Only very recently, WHO has endorsed new molecular platforms for resistance prediction, such as Truenat MTB (Molbio Diagnostics, India), Abbott RealTime MTB RIF/INH (Abbott, USA), cobas MTB-RIF/INH (Roche, Switzerland) [2]. Importantly, WGS has already been employed for routine drug resistance prediction by several TB laboratories in Europe [3, 4]. Moreover, the first WHO-endorsed catalogue of drug resistance-related mutations in MTBC has prompted the implementation of WGS at the national level [5].

Among WHO-approved, conventional approaches for MTBC DST, the most common are the proportion method on Löwenstein-Jensen (LJ) medium and the Bactec MGIT 960 System (MGIT) (Becton Dickinson, USA). In both methods, drug susceptibility profile is established based on one CC of the drug, which yields a qualitative result. DST measuring the range of MIC values, and thus providing quantitative results is still not a routine practice in TB diagnostics.

Both conventional, phenotypic, and genotypic methods produce discordant results, leading to disparate interpretations and conflicting conclusions [6–8]. DST based on mutation profiling is limited by new mutations, different from those targeted by the assay or failure in detection of low frequency mutation variants [8, 9]. Whereas qualitative phenotypic methods do not meet current diagnostic needs due to long turnaround time and flaws associated with the CC used. Resistance mechanisms in MTBC often cause MIC distributions to overlap with those of

susceptible strains. This overlapping scenario leads to a lack of reproducibility of CC DST, as the CC intersects with the MIC distributions of strains harbouring these resistance mechanisms [7, 10–12]. Cases with discrepant DST results are usually subject to semi-empiric treatment often leading to unfavorable outcomes [13, 14]. Sequencing data coupled with MICs might be a powerful tool for more accurate detection of drug resistance in MTB. However, neither is available for routine diagnostics in Poland and Lithuania. Regarding conventional molecular techniques, GeneXpert MTB/RIF (Cepheid, USA) coupled with LPA is most widely used in the region. A relatively few studies have investigated comparatively the efficacies of phenotype- and molecular-based methods for drug resistance prediction among MTBC strains from Central and Eastern Europe [15–17]. Therefore, the aim of this study was to compare conventional DST methods, both CC and MIC DST with molecular (LPA and WGS-based) DST methods in terms of their ability to detect RIF/INH resistance in MTB. The impact of individual mutations identified by WGS analysis on MIC level was also assessed.

Methods

Study sample

This study was carried out on 208 *M. tuberculosis* strains (130 MDR; 78 drug susceptible, DS), collected from as many patients in Lithuania ($n=122$) and Poland ($n=86$) between 2018 and 2021.

Primary isolation, culturing, species identification, and DST were performed with standard mycobacteriological procedures [18, 19].

Phenotypic DST

Qualitative methods (CC DST)

Conventional DST was performed for all tested strains with either MGIT 960 System (Becton Dickinson, USA) ($n=175$) or LJ medium ($n=33$), following the WHO recommendations [19]. The CCs for RIF were 1.0 mg/L (MGIT) and 40 mg/L (LJ), and those for INH were 0.1 mg/L (MGIT) and 0.2 mg/L (LJ). For comparison purposes, the results from both MGIT and LJ methods were combined.

Quantitative method (MIC DST)

DST using the broth microdilution method was performed for 185 strains (124 MDR; 61 DS). The Sensititre MYCOTBI® plates (Thermo Fisher Scientific, USA) were used for MIC determination. The inocula were prepared according to the manufacturer's instructions, and the plates were incubated at 35–37 °C until day 21 in ambient

air. The MIC readings were taken after 10, 14, and 21 days, using an inverted mirror and read as the lowest concentration of the drug yielding no visible growth. The MICs taken after 14 days were used for the analytical purposes. The MIC values were presented as a mode with a corresponding range. The MIC DST method was evaluated using the MIC breakpoints proposed by the Clinical and Laboratory Standards Institute (CLSI), i.e. ≤ 0.5 $\mu\text{g/mL}$ for RIF and ≤ 0.12 $\mu\text{g/mL}$ for INH [20].

Molecular DST

LPA

LPA (GenoType MTBDRplus ver. 2.0, HAIN, Germany) was carried out on all 208 strains and in accordance with the manufacturer's instructions. Interpretative criteria defined by the Global Laboratory Initiative and WHO were followed [21]. Strains exhibiting inferred resistance to RIF/INH were considered as resistant. Mutations in the *rpoB* gene were assigned using the *E. coli* numbering system, corresponding to point mutations identified with a WGS-based pipeline with a distance of minus 81 bp [22].

WGS-based methods for prediction of drug resistance

DNA extraction

Genomic DNA was extracted from material cultured on LJ using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) ($n=86$) or using a modified cetyltrimethylammonium bromide method ($n=122$), as described elsewhere [23]. The purified DNA was dissolved in TE buffer and quantified with the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

Whole-genome sequencing and raw sequencing data processing

Paired-end libraries were prepared from high-quality genomic DNA with the NovaSeq 6000 Reagent Kits according to the manufacturer's instructions (Illumina, USA). WGS was done with Illumina NovaSeq 6000 sequencer (Illumina, USA) in 2×150 bp paired-end mode. The sequencing yielded in acquisition of 1.18 to 5.16 of raw Gbps (median 2.34 Gbps) per strain. Processing of sequencing data was carried out, as described previously [24]. The reconstructed genomes consisted of 52 to 102 contigs (median: 76) of total length of 4.33 to 4.53 Gbps (median: 4.37 Gbps), coverage ranging between 269 to 1180x (median: 534x) and presented 99.61 to 99.94 and 0 to 0.67 genome completeness and contamination levels.

WGS-based drug resistance profiling

The analysis of drug-resistant profiles, based on WGS data, was performed in two parallel ways. First, genes

related with INH/RIF resistance from WHO MTBC mutations catalogue (WHO/UCN/MTB/PCI/2021.7) were recovered from the reference *M. tuberculosis* H37Rv strain (NCBI RefSeq accession number: NC_000962.3) (Additional file 1: Table S1). Then, all corresponding regions from genome assemblies were aligned with the reference sequences with MAFFT v7.490-1 to identify mutations.

The second approach included the application of *in silico* drug resistance prediction tools, i.e. TB Profiler v4.0.1 (database version: 9929954) and Mykrobe v0.10.0. These were run locally using subsets of 3,000,000 pairs of reads per genome to retain similar coverage (ca. 100x) of analyzed genomes.

Data analysis

The performance of GenoType MTBDRplus ver. 2.0 and WGS for drug resistance prediction was measured using sensitivity, specificity, positive and negative predictive values, assuming CC DST results as the reference standard. Data were analyzed using Microsoft Excel ver. 2311 and OpenEpi ver. 3.01.

Results

CC and MIC DST

CC DST was carried out on 208 MTBC strains. A total of 130 (130/208; 62.5%) strains were resistant to RIF/INH, as per MGIT and proportion method (Additional file: Table S2), thus meeting the MDR definition. Furthermore, 185 strains had their MICs determined using MYCOTBI plates and were stratified by CC DST results (Fig. 1). The mode MICs were >16 $\mu\text{g/mL}$ (≤ 0.12 – >16 $\mu\text{g/mL}$) and ≤ 0.12 $\mu\text{g/mL}$ (≤ 0.12 – 0.5 $\mu\text{g/mL}$) for RIF-resistant and RIF-susceptible strains, respectively. As for INH, the MICs for INH-resistant strains varied widely (mode MIC of 2 $\mu\text{g/mL}$; ≤ 0.03 – >4 $\mu\text{g/mL}$), whereas for INH-susceptible strains the MICs were within a narrow range of 0.03–0.06 $\mu\text{g/mL}$ (mode MIC of ≤ 0.03 $\mu\text{g/mL}$).

Molecular DST

LPA

LPA identified 125 strains as MDR (Additional file 1: Table S2). Resistance to RIF was detected in 128 (128/208; 61.5%) strains. The most prevalent mutation pattern was S531L (MUT3) found in 108 (108/208; 51.9%) strains, coupled with either Δ WT8 (S531L; 107/208; 51.4%) or with a WT8 band present (S531L; 1/208; 0.5%).

Resistance to INH was detected in 125 (125/208; 60.1%) strains (Additional file 1: Table S2). All but two were found to carry mutations in the codon 315 of the *katG* gene. Of these, the majority (122/208; 58.7%) had S315T1 (MUT1) with a WT band present (4/208; 1.9%)

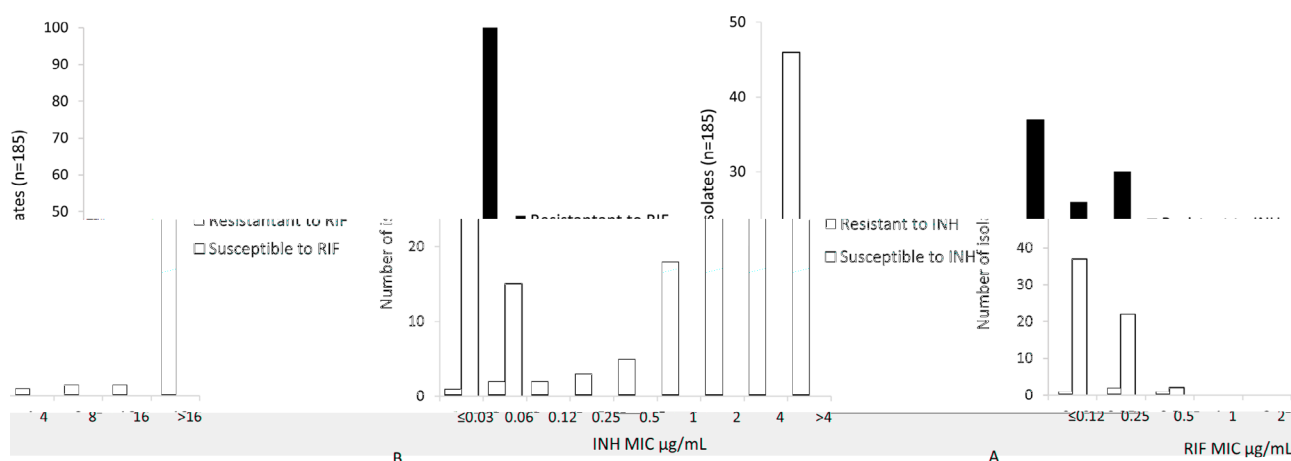


Fig. 1 Distribution of RIF (A)/INH (B) MICs stratified by CC DST results. *RIF* rifampicin; *INH* isoniazid; *MIC* minimum inhibitory concentration; *DST* drug susceptibility testing; *CC* critical concentration

Table 1 Comparison of MIC DST and CC DST results for RIF/INH

MGIT/LJ CC [µg/mL]	No. of strains		MYCOTBI MIC [µg/mL]	Interpretation of MYCOTBI	Agreement [%]
	S	R			
RIF 1.0/40.0	61	4*	≤ 0.5	S	97.8
	0	120	> 0.5	R	
INH 0.1/0.2	61	5	≤ 0.12	S	97.3
	0	119	> 0.12	R	

DST drug susceptibility testing; *RIF* rifampicin; *INH* isoniazid; *MGIT* Bactec MGIT 960 system; *LJ* Löwenstein-Jensen; *CC* critical concentration; *MIC* minimum inhibitory concentration; *MYCOTBI* 96-well MYCOTBI® plates for MIC testing (ThermoFisher Scientific, USA); * – 2/4 strains became RIF-resistant after additional week of incubation on day 21; *S* susceptible; *R* resistant

or absent (118/208; 56.7%), followed by one (1/208; 0.5%) strain with both WT and MUT bands absent.

Mutations in the *inhA* gene (19/208; 9.1%) were rare; 17 (17/208; 8.2%) strains harboured single C-15T polymorphism, one (1/208; 0.5%) strain had T-8 C polymorphism, and one (1/208; 0.5%) missed both WT1 and MUT bands in -15 region of the *inhA* promoter.

Mutation profiling by WGS

A total of 124 (124/208; 59.6%) strains were identified as MDR using WGS (Additional file 1: Table S2). Overall, 125 (125/208; 60.1%) strains were found RIF-resistant, while 124 (124/208; 59.6%) were INH-resistant.

The most prevalent RIF resistance conferring mutation was *rpoB* S450L accounting for a total of 105 (105/208; 50.9%) strains, while the remaining mutations were as follows: *rpoB* H445D (6/208; 2.9%), *rpoB* D435V (5/208; 2.4%), *rpoB* H445Y (3/208; 1.4%), *rpoB* S450W (3/208; 1.4%), *rpoB* H445L (1/208; 0.5%), *rpoB* H445C (1/208; 0.5%), *rpoB* H445N (1/208; 0.5%) (Additional file 1: Table S2).

The majority (124/208; 59.6%) of INH-resistant variants harboured single *katG* S315T (106/208; 51.0%) mutation. The remaining strains appeared to be double mutants containing *katG* S315T and either *inhA* C-15T (15/208; 7.2%) or *inhA* T-8 A (1/208; 0.5%) substitution.

Single *inhA* C-15T substitution occurred in two (2/208; 1%) strains only (Additional file 1: Table S2).

Comparison of methods

CC DST vs. MIC DST

Overall, the agreement between CC and MIC DST was equally high for RIF (97.8%) and INH (97.3%) (Table 1). Only 4 (4/185; 2.2%) strains gave discordant results for RIF exhibiting low MICs for strains DLT80 (≤ 0.12 µg/mL), KR-PLM-15 (0.5 µg/mL), DLT48, and DLT76 (0.25 µg/mL). Noteworthy, the latter two strains (DLT76 and DLT48) required additional week of incubation to reach higher MICs of > 16 µg/mL and 1 µg/mL, respectively. A total of 5 (5/185; 2.7%) strains demonstrated discordant results for INH. Two of these strains (DLT33, DLT107) exhibited MICs of 0.12 µg/mL, while another two (KR-PLM-15, DLT49) had MICs of 0.06 µg/mL. One strain (DLT80) had an MIC of ≤ 0.03 µg/mL.

Sequence analysis vs. software platforms

The results of drug resistance profiling performed with WGS and two software platforms (TB Profiler and Mykrobe) showed a concordance of 100% for RIF and 99% for INH (Table 2). The latter result was due to two strains that were identified as INH-susceptible upon WGS, yet resistant by either TB Profiler and Mykrobe (G48A

Table 2 Performance characteristics of different methods and tools for the prediction of resistance to RIF/INH

Methods/tools compared					Sensitivity (%), CI	Specificity (%), CI	PPV (%), CI	NPV (%), CI	Diagnostic accuracy (agree- ment), (%), CI	Kappa- value
MYKROBE/TB Profiler vs. WGS										
Drug	*B – R ◆UT– R	*B – S ◆UT– S	*B – R ◆UT– S	*B – S ◆UT– R						
RIF	125	83	0	0	100 (97–100)	100 (95.6–100)	100 (97.02–100)	100 (95.6–100)	100 (98.2–100)	1
INH	124	82	0	2†	100 (97–100)‡ 100 (97–100)§	97.6 (91.7–99.3)‡ 98.8 (93.5–99.8)§	98.4 (94.4–99.6)‡ 99.2 (95.6–99.9)§	100 (95.5–100)‡ 100 (95.5–100)§	99.04 (96.6–99.7)‡ 99.52 (97.3–99.9)§	0.98‡ 0.9899§
LPA (GenoType MTBDRplus ver. 2.0) vs. CC DST										
	LPA R pDST R	LPA S pDST S	LPA R pDST S	LPA S pDST R						
RIF	128	78	0	2	98.5 (94.6–99.6)	100 (95.3–100)	100 (97.1–100)	97.5 (91.3–99.3)	99.04 (96.6–99.7)	0.9796
INH	125	78	0	5	96.2 (91.3–98.4)	100 (95.3–100)	100 (97–100)	94 (86.7–97.4)	97.6 (94.5–98.97)	0.9494
WGS vs. CC DST										
	WGS R pDST R	WGS S pDST S	WGS R pDST S	WGS S pDST R						
RIF	125	78	0	5	96.2 (91.3–98.4)	100 (95.3–100)	100 (97–100)	94 (86.7–97.4)	97.6 (94.5–99)	0.9494
INH	124	78	0	6	95.4 (90.3–97.9)	100 (95.3–100)	100 (97–100)	93 (85.3–96.7)	97.12 (93.85–98.7)	0.9394
LPA (GenoType MTBDRplus ver. 2.0) vs. WGS										
	LPA R WGS R	LPA S WGS S	LPA R WGS S	LPA S WGS R						
RIF	124	79	4	1	99.2 (95.6–99.9)	95.2 (88.3–98.1)	96.9 (92.2–98.8)	98.8 (93.3–99.8)	97.6 (94.5–99)	0.9496
INH	124	83	1	0	100 (97–100)	98.8 (93.6–99.8)	99.2 (95.6–99.9)	100 (95.6–100)	99.5 (97.3–99.9)	0.99

RIF rifampicin; INH isoniazid; PPV positive predictive value; NPV negative predictive value; CI confidence intervals; *B – bioinformatic pipeline; ◆UT – user friendly tools (Mykrobe and TB Profiler); †resistance to INH predicted by Mykrobe only; ‡Mykrobe only; §TB Profiler only; pDST phenotypic drug susceptibility testing; WGS whole-genome sequencing pipeline; LPA line-probe assay; S susceptible; R resistant

mutation in the promoter of the *ahpC* gene; 1 strain) or Mykrobe (L302L mutation in the *fabG1* gene; 1 strain).

Performance of molecular methods

The overall performance of LPA was satisfactory with a sensitivity of 98.5% and 96.1%, and a specificity of 100% for detecting resistance to RIF and INH, respectively (Table 2). Nevertheless, LPA failed to detect resistance in 5 (5/130; 3.8%) phenotypically INH-resistant and two (2/130; 1.5%) RIF-resistant strains.

Compared to LPA, WGS demonstrated slightly lower sensitivity (96.2%) for RIF and (95.4%) for INH, while the specificity for both drugs was exactly the same (100%). Interestingly, *rpoB* S450L (strains DLT80, DLT48, DLT76), *rpoB* D435V (strain D-PL-22), and *katG* S315T (strain DLT80) mutations were not detected by WGS, though they were determined by LPA. The concordance between LPA and WGS was 97.6% and 99.5% for RIF and INH, respectively (Table 2).

Phenotypic vs. molecular methods

A total of 11 (11/208; 5.3%) MDR strains (determined by CC DST) had discordant results with either WGS, LPA or MIC DST. The discordance concerned resistance to RIF in two (2/208; 1%) strains, INH – in 5 (5/208; 2.4%)

strains, and to both drugs – in four (4/208; 1.9%) strains (Fig. 2).

The LPA failed to detect RIF-resistance in strain (DLT102) carrying *rpoB* S450W. However, this was not observed for the other two *rpoB* S450W mutants (DLT41, DLT89). In case of DLT41 and DLT89 strains, mutation *rpoB* S450W resulted in absence of both *rpoB* WT8 and MUT bands which indicates inferred resistance. However, the WT8 band was present in strain DLT102 which let it to be considered as RIF wild type.

As for the strain KR-PLM-15, a MIC testing revealed possible flaws with the CC DST, as the MICs for RIF and INH only increased slightly reaching 0.5 µg/mL and 0.06 µg/mL. Moreover, no resistance-conferring mutations were detected by LPA and WGS.

One *katG* S315T mutant (DLT49) increased MIC only to 0.06 µg/mL, possibly due to technical errors, as CC DST results were congruent with LPA/WGS. No INH-resistance associated mutations were detected by LPA/WGS for strains KR-PLM-8 and KR-PLM-20. For strain KR-PLM-8, both CC and MIC DST results were congruent. A similar comparison for strain KR-PLM-20 was not possible due to contamination with non-tuberculous mycobacteria.

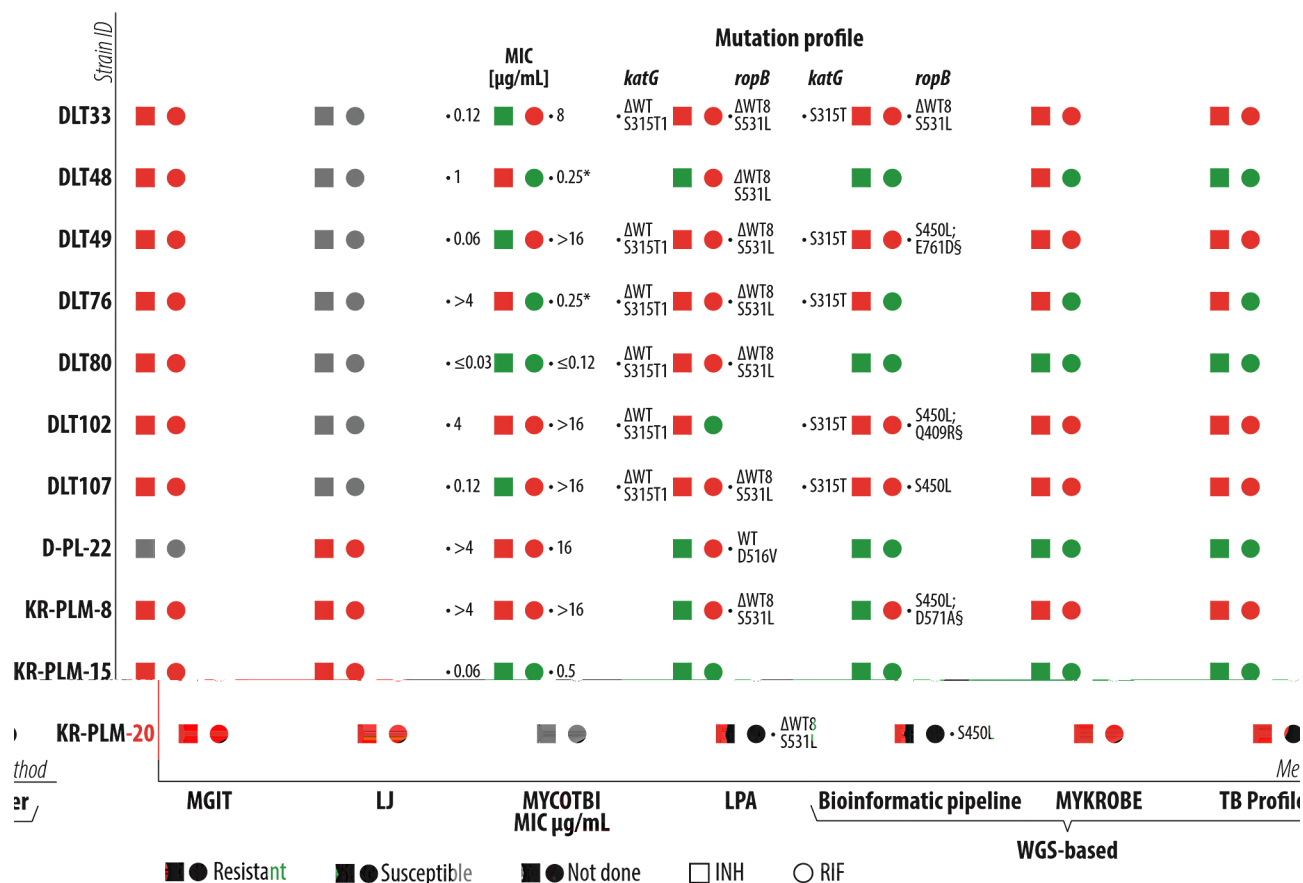


Fig. 2 Phenotypic and genotypic drug susceptibility profiles of RIF/INH in 11 strains with discordant results. *RIF* rifampicin; *INH* isoniazid; *MGIT* Bactec MGIT 960 system; *LJ* Löwenstein-Jensen; *MIC* minimum inhibitory concentration; *MYCOTBI* 96-well MYCOTBI® plates for MIC testing (ThermoFisher Scientific, USA); The MICs taken after 14 days were used for the Fig. 3; *LPA* line-probe assay; *WT* wild type; Δ – absence of hybridization signal with wild-type probes; *WGS* whole-genome sequencing; § – mutations of uncertain significance; * – strains became RIF-resistant after additional week of incubation on day 21

Strains for which resistance was detected by LPA but not by WGS (DLT80; DLT48) had MICs below the CLSI breakpoints for RIF/INH (Fig. 2).

MIC DST vs. WGS

Among RIF-resistant strains, all but two (DLT33–8.0 μg/mL, DLT60–16.0 μg/mL) with *rpoB* S450L mutation (97/185; 52.4%) had their MICs >16 μg/mL, as well as strains harbouring the *rpoB* H445D (6/185; 3.2%), H445Y (3/185; 1.6%), and S450W (3/185; 1.6%) mutations (Fig. 3). Five (5/208; 2.4%) strains with *rpoB* D435V mutation had their MICs spanned over a wide range of values (i.e. from 4.0 μg/mL to >16 μg/mL). So far, seven mutations conferring so-called borderline RIF-resistance, expected to exhibit lower MIC values, have been identified [11]. Two such mutations (*rpoB* H445L and H445N) were detected and resulted in noticeably high MICs of 4.0 μg/mL and >16 μg/mL, respectively. Single *rpoB* H445C mutant (1/185; 0.5%) had MIC of 8.0 μg/mL. Single genotypically susceptible strain (1/185; 0.5%) demonstrated MIC of 16 μg/mL. The MICs of the remainder of

the genotypically RIF-susceptible (65/185; 35.1%) strains fell within a range of ≤0.12–0.5 μg/mL.

The MIC distribution among INH-resistant strains harbouring *katG* S315T mutation (103/185; 55.7%) ranged from 0.06 to >4 μg/mL, with a mode MIC of 2 μg/mL. The mode MIC was two-fold higher (>4 μg/mL) among double mutants harbouring *katG* S315T and either *inhA* C-15T (14/185; 7.6%) or *inhA* T-8 A (1/185; 0.5%) mutation compared to single *katG* mutants. Single (1/185; 0.5%) *inhA* C-15T promoter mutant had its MIC of 1 μg/mL. The MIC of genotypically INH-susceptible strains ranged from ≤0.03 to 0.6 μg/mL (63/185; 34.0%) with three strains showing unexpectedly high MICs of 1 μg/mL (1/185; 0.5%) and >4 μg/mL (2/185; 1.1%).

Discussion

The accuracy of conventional DST for MTBC strains is influenced by several factors including technical errors, intrinsic limitations related to the overlap of MIC distributions between susceptible and resistant strains, and prevalence of resistance-associated mutations [7, 10–12].

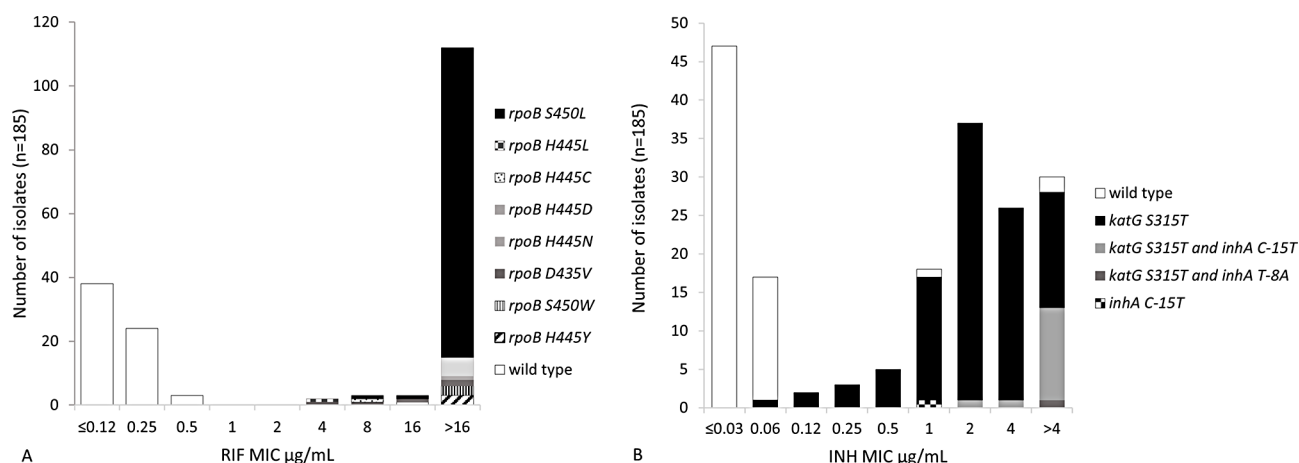


Fig. 3 Distribution of RIF (A)/INH (B) MICs stratified by the presence of resistance-conferring mutations (WGS). RIF rifampicin; INH isoniazid; MIC minimum inhibitory concentration; DST drug susceptibility testing; CC critical concentration; WGS whole-genome sequencing

Moreover, qualitative methods are labour- and time-consuming. MIC DST is more informative, guiding in selecting the most suitable treatment regimen [7, 11]. However, for all phenotypic DST tests biosafety level 3 is applied, while molecular techniques require minimum biosafety measures. Rapid molecular assays, such as LPA predict RIF/INH resistance efficiently and in a timely manner, yet they cover only the most frequently occurring mutations. The utility of WGS is currently limited by the need of highly qualified staff, and sophisticated bioinformatic procedures, therefore freely accessible tools could become beneficial, if updated timely. Despite the proven cost-effectiveness of routine WGS [3, 25], it cannot entirely replace conventional DST, as the functionality of some resistance genes can be confirmed by phenotypic methods only [26, 27]. Numerous studies have previously demonstrated strains exhibiting the so-called low-level RIF resistance, currently known as the borderline resistance [6, 11, 12, 28, 29]. In 2021, the WHO announced the urgent need to change RIF CC from 1.0 mg/L to 0.5 mg/L using MGIT and Middlebrook 7H9 medium. The discussion, however, persists whether this newly proposed CC is optimal or should be further lowered [13]. Our results are supportive to the latter, as nearly all susceptible strains had their MICs below 0.5 mg/L with only two strains exhibiting exactly this value. The MIC overlap was observed for four strains, of which two required an additional week of incubation to reach a higher MIC, resulting in only two exhibiting discordant results. Previous studies have also reported the need for an additional week of incubation for particular strains or drugs [30, 31]. The interpretation of congruency between CC and MIC DST for INH was challenging as its CC is not represented by an exactly equivalent MIC value in the 96-well plate. According to the CLSI, the MIC breakpoint of 0.12 µg/mL should be used, although CRyPTIC consortium has proposed lower epidemiological cut-off value of 0.1 µg/

mL, which is closer to the INH CC in MGIT (0.1 µg/mL) recommended by WHO [19, 20, 28]. Two strains exhibiting this exact value (0.12 µg/mL) were INH-resistant in MGIT, hence decreasing the concordance between the two methods. As our study started in 2018, there was no established reference method for MIC determination in MTBC set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) subcommittee on antimycobacterial susceptibility testing (published in 2019) or WHO documents on MIC (published in 2022) [32, 33]. Consequently, technical inconsistencies between the manufacturer's manuals and CLSI standard may have contributed to the observed discrepancies.

The highest concordance with MGIT/LJ results was shown for LPA (sensitivity: RIF – 98.46%; INH – 96.15%), superseding that of WGS (sensitivity: RIF – 96.15%; INH – 95.38%), although specificity for both molecular approaches reached 100%. Surprisingly, in certain strains mutations such as *rpoB* S450L (S531L), *rpoB* D435V (D516V) or *katG* S315T were detected by LPA, but not by WGS. We could not explain these discrepancies, and it was not observed in other studies [34, 35]. However, previous data have shown that wild type strains upon sequencing could have absent WT probes in LPA [36] which would be interpreted as inferred resistance. We found that three RIF-resistant (with *rpoB* S450W) and one INH-resistant (*katG* S315T) strains were non-reactive with MUT probes coupled with absent or present WT band. In previous studies, *rpoB* S450W has been reported as the most prevalent mutation associated with inferred RIF-resistance [35, 36]. Therefore, the inclusion of the *rpoB* S450W mutant probe would be recommended.

WGS and LPA showed that the majority of RIF-resistant strains carried *rpoB* S450L while most of the INH-resistant strains harboured *katG* S315T. These substitutions are the commonest resistance-associated

mutations [10, 37]. The other strains also contained well-characterised mutations listed in the WHO's mutation catalogue. In this study, Mykrobe and TB Profiler performed equally well at predicting RIF/INH resistance (sensitivity 100%; specificity 100% for RIF and 97.6–98.8% for INH), which is in line with previous data [38, 39]. Discrepancies for KR-PLM-8 strain occurred due to the G48A mutation in the promotory region of *ahpC*, which was detected by both software platforms. However, only the *ahpC* structural gene sequence, and not its regulatory region should be interrogated for INH resistance mutations, according to the WHO's mutation catalogue [37]. Furthermore, the bioinformatic pipeline did not cover the *fabG1* gene, which accounted for INH resistance in strain DLT48, according to the Mykrobe software. The congruency of different software tools with conventional methods depends on regular updates, which might influence different findings over time [38, 39]. Although molecular mechanisms behind resistance to RIF/INH are fairly understood, in different geographic regions discordance rates between phenotypic and genotypic tests may vary from 5 to 15% due to limitations of the methods [6–8]. Given that initially DST is performed only for the first-line drugs and subsequently for the second-line drugs, uncertainties due to discordant results for RIF/INH are of critical importance. Misclassification of susceptible strains as resistant ones exposes patients to prolonged, less effective, and more toxic treatment with MDR-TB regimen, and may result in the development of further drug resistance [14].

The performance of different DST methods for the detection of resistance to INH/RIF in Lithuania and Poland has been understudied. We identified 11 (5.3%) strains for which at least one discrepancy occurred with different modalities used for determination of resistance to RIF and/or INH. Three inconsistencies were due to technical flaws and limitations of the methods (i.e. *rpoB* S450W mutation not covered by LPA in one strain and false INH-resistance detected by software tools in two strains). The reason behind other discrepancies may be more complex and relate to different growth rates and heteroresistance. Furthermore, the presence of heteroresistant strains at low frequencies have been shown to produce susceptible phenotype upon DST [9]. Contaminants, previously undescribed mutations, and LPA interpretation errors might also be explicative of discordant results [11].

The mode MIC of >16 µg/mL for RIF was observed for nearly all *rpoB* S450L mutants along with the *rpoB* S450W, H445D, H445Y mutants. The *rpoB* S450L polymorphism has been widely recognized as considerably increasing MIC of RIF [10, 28, 37]. In contrast, in five strains, *rpoB* D435V was detected, a mutation which has previously been reported to confer a moderate level of

RIF resistance [40]. Nevertheless, all five *rpoB* D435V mutants had a broad MIC distribution (4–>16 µg/mL). Two borderline resistance harbouring strains, with either *rpoB* H445L or H445N alteration, had MICs of 4 µg/mL and >16 µg/mL, respectively. MIC distribution of INH mutants ranged broadly from 0.06 to >4 µg/mL, which could be explained by different underlying resistance mechanisms. The broader range of MICs for *katG* mutants compared to *inhA* promoter mutants has already been reported [41]. Here, the mode MIC of double *katG* and *inhA* mutants was one dilution greater (≥ 4 µg/mL; 2– ≥ 4 µg/mL) compared to single *katG* mutants (2 µg/mL; 0.06– ≥ 4 µg/mL). Irrespective of discordant results, the MICs of genotypically INH-susceptible strains fell within the range of ≤ 0.03 –0.06 µg/mL, which was in line with the EUCAST and CLSI proposed MIC breakpoint (≤ 0.12 µg/mL) [20, 32], albeit one dilution lower.

There were several limitations to this study. First, in MGIT testing we used RIF CC of 1.0 mg/L, instead of 0.5 mg/L, which was endorsed by WHO in early 2021 [12]. Therefore, we possibly missed some of the borderline resistance-conferring mutants. Furthermore, MYCOTBI plates have been criticized by WHO and EUCAST experts over its improper inoculum preparation and limitations related to truncated MICs or not specified lower ends of quality control ranges [33]. Sub-optimal reading days were indicated among other concerns. It was advised to perform the readings on days 7, 14, and 21 instead of 10 and 21, as indicated in the manufacturer's protocol [33].

Conclusions

- Considering MIC DST results, current CCs of the first-line anti-TB drugs might be inappropriately high and may need to be revised.
- The RIF-resistant strains demonstrated a relatively homogenous MIC distribution while for INH-resistant strains MIC values fell within a broad range.
- The discordance rate of DST results between different methods was calculated at 5.3%. Technicalities, previously undescribed mutations, and intrinsic resistance are among plausible causes of discordant results.
- Currently available software platforms provide satisfactory performance in predicting resistance to RIF/INH. Thus, adopting the software-based WGS tools may improve timely diagnosis of MDR-TB in Poland, Lithuania, and other countries where the WGS-based approach has not yet been applied.

Abbreviations

CC	critical concentration
CLSI	Clinical and Laboratory Standards Institute

DST	Drug susceptibility testing
DS	Drug susceptible
EU/EEA	European Union/European Economic Area
EUCAST	European Committee on Antimicrobial Susceptibility Testing
INH	Isoniazid
LJ	Löwenstein–Jensen
LPA	Line probe assay
MGIT	Bactec MGIT 960 system
MIC	Minimum inhibitory concentration
MTBC	Mycobacterium tuberculosis complex
RIF	Rifampicin
RR/MDR-TB	Rifampicin-resistant/multi-drug resistant tuberculosis
TB	Tuberculosis
WGS	Whole-genome sequencing
WHO	World Health Organization

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

LV drafted the manuscript, performed the laboratory experiments for strains from Lithuania including a subset from Poland, and analyzed the laboratory data; ZB co-drafted the manuscript, extracted mycobacterial DNA, performed MIC DST for strains from Poland, and analyzed the laboratory data; MP performed LPA for strains from Poland; DB extracted mycobacterial DNA for all strains from Lithuania; PD, MD performed bioinformatic analysis; TK made revisions to the initial version of the manuscript; BN, EVD, LV, RK collected and revised patient data; BN, EVD, EV, PS obtained approval for this study; EVD, EV were responsible for the patient enrollment. TJ, PS, EV conceived and designed the experiments; TJ revised the manuscript and secured funding for its publication.

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Data availability

The raw reads were deposited under NCBI Bio-Project, accession number PRJNA931475.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical University of Warsaw's Bioethics Committee and Lithuanian Regional Biomedical Research's Ethics Committee. The requirement for informed consent from the study subjects was waived by the Medical University of Warsaw's Bioethics Committee and Lithuanian Regional Biomedical Research's Ethics Committee since the study sample was collected during routine clinical practices, and all personal data were anonymized prior the study. All experimental protocols and methods were approved by the Medical University of Warsaw's Bioethics Committee and Lithuanian Regional Biomedical Research's Ethics Committee. All methods were carried out in accordance with guidelines and regulations of the Medical University of Warsaw, Vilnius University, and Vilnius University Hospital Santaros klinikos.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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