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Drug susceptibility testing of *Mycobacterium tuberculosis* using next generation sequencing and Mykrobe software

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Abstract

Introduction. *Mycobacterium tuberculosis* is the causative agent of tuberculosis. Drug susceptibility testing is performed by phenotypic and molecular tests. Commonly used for phenotypic drug susceptibility testing is the automated BACTEC system in a liquid culture medium. Drug susceptibility by line probe molecular tests was introduced almost 15 years ago. Recently whole genome sequencing (WGS) analysis of *M. tuberculosis* strains demonstrated that genotyping of drug-resistance could be accurately performed. Several software tools were developed.

Our study **aimed** to perform whole-genome sequencing on phenotypically confirmed multi-drug resistant (MDR) *M. tuberculosis* strains, to identify drug-resistant mutations and to compare whole-genome sequencing profiles with line probe assay and phenotypic results.

Materials and methods. We performed analysis on 34 MDR *M. tuberculosis* Bulgarian strains. Phenotypic drug susceptibility testing was performed on the BACTEC system. For molecular testing of drug susceptibility to first- and second-line tuberculostatics, we applied line probe assay Geno Type MTBDR *plus* v.1.0 и Geno Type MTBDR *sl* v.1.0. Sequencing was performed on MiSeq. Generated FASTQ files were analyzed for known drug-resistant mutations with the software platform Mykrobe v.0.8.1.

Results. All three methods — phenotypic analysis using the BACTEC system, genetic analysis of strains applying the Geno Type test and Mykrobe software gave comparable sensitivity/resistance results for the studied strains. All phenotypically proven rifampicin and isoniazid-resistant strains were 100% confirmed using Mykrobe software. The C-15T mutation is a marker for isoniazid resistance in strains of the SIT41 spoligotype. We observed a 75% (21/28) agreement between BACTEC and Mykrobe for ethambutol resistance. Phenotypically, 87% (n = 27) of the strains are resistant to streptomycin, but only 59% (n = 19) are proven by Mykrobe software. Comparing phenotypic and genotypic resistance to ofloxacin, amikacin and kanamycin, we observed 100% coincidence of results.

Conclusions. Whole-genome sequencing approach is relatively expensive and laborious but useful for detailed analysis such as epidemiological genotyping and molecular drug susceptibility testing.

Keywords: *M. tuberculosis, FASTQ, next-generation sequencing, drug resistance*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ministry of Health of the Republic of Bulgaria (Protocol No. 7, August 2, 2019 on the conditions and procedures for conducting diagnosis, prevention and control of tuberculosis).

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Определение чувствительности *Mycobacterium tuberculosis* к противотуберкулёзным препаратам с помощью полногеномного секвенирования и программного обеспечения «Mykrobe»

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Аннотация

Введение. Чувствительность *Mycobacterium tuberculosis* к противотуберкулёзным препаратам устанавливается с помощью фенотипических и молекулярных методов. Анализ целого генома штаммов *M. tuberculosis* даёт возможность предсказывать резистентность к лекарствам для большого числа медикаментов. Для этого разработано несколько видов программного обеспечения.

Цель работы — определить чувствительность *M. tuberculosis* к антитуберкулёзным препаратам с помощью фенотипического и генотипического анализа, а также полногеномного секвенирования с использованием программного обеспечения «Mykrobe».

Материалы и методы. Исследовали 34 мультирезистентных штамма *M. tuberculosis*, выделенных из клинических материалов 34 пациентов в Болгарии. Все они были подтверждены фенотипически с помощью «BACTEC MGIT 960 System». Для определения резистентности к противотуберкулёзным средствам первого и второго ряда пользовались тестами для линейной гибридизации «Geno Type MTBDR *plus* v.1.0» и «Geno Type MTBDR *sl* v.1.0». Штаммы *M. tuberculosis* секвенировали с помощью «MiSeq». Для электронной резистограммы применяли программное обеспечение «Mykrobe v.0.8.1».

Результаты. Все три метода — фенотипический анализ, генетический анализ и электронная резистограмма с помощью программного обеспечения «Mykrobe» — дали сопоставимые результаты чувствительности/резистентности исследуемых штаммов. Все фенотипически доказанные штаммы, резистентные к рифампицину и изониазиду, были подтверждены на 100% с помощью программного обеспечения «Mykrobe». Мутация C-15T является маркером для резистентности к изониазиду у исследуемых нами штаммов со сполиготипом SIT41. Мы наблюдали 75% (21/28) совпадения результатов по «BACTEC» и «Mykrobe» в отношении резистентности к этамбутолу. Фенотипически 87% (*n* = 27) штаммов были устойчивы к стрептомицину, и лишь 59% (*n* = 19) доказаны программным обеспечением «Mykrobe» как таковые. Сравнивая фенотипическую и генотипическую резистентность к офлоксацину, амикацину и канамицину, мы наблюдали совпадение результатов на 100%.

Выводы. Секвенирование целого генома относительно дорого и трудоёмко, но представляет собой ценный инструмент эпидемиологического генотипирования и определения восприимчивости к лекарственным средствам.

Ключевые слова: *M. tuberculosis, FASTQ, секвенирование следующего поколения, лекарственная резистентность*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Министерством здравоохранения Республики Болгария (Постановление № 7 от 02.08.2019 об условиях и процедурах проведения диагностики, профилактики и борьбы с туберкулезом).

Источник финансирования. Исследование финансировалось Болгарским национальным научным фондом (гранты № ДН13 / 4-15.12.2017 и ДН13 / 1-14.12.2017).

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Drug resistance is a serious problem challenging antimicrobial therapy around the world[1]. Treatment of tuberculosis patients with antituberculosis drugs is one of the main strategies for disease control in Bulgaria and worldwide [1–4]. *Mycobacterium tuberculosis* infection and resistance in some cases is associated with coinfection like HIV [5–7], and this fact should be taken into account when therapy is subscribed. Antimicrobial drugs gradually lose activity against pathogens as a result of increasing microbial resistance. Along with classical methods of determination of *M. tuberculosis* complex drug resistance, new ones related to DNA sequence assays were recently introduced[2]. PCR and targeted sequencing of genes causing antimicrobial resistance are widely used and became common. The introduction of next-generation sequencing technologies gave to researchers new opportunities for more powerful and detailed analysis [8–11]. Another advantage of NGS analysis is that when a strain once being sequenced, the information could be stored in formats such as FASTQ, FAST5 or others, depending on the technology, this strain can be analyzed in further studies with different software tools for different genetic, phylogenetic and epidemiological investigations. Illumina sequencing technology is widely applied. Soon application of next-generation sequencing technology will be mandatory in the description of bacterial or viral strains and will be widely used in other fields of medicine and will displace many phenotypic and current molecular genetic methods. Software tools were developed for drug resistance determination using data of whole-genome sequenced microorganisms, such as ABRicate, ARIBA [12], ARGs-OAP [12], ARG-ANNOT [14], CASTB [15], KvarQ [16], MTBseq [17], PhyResSe [18], RAST [19], ResFinderST [20], RGI [21], SRST2 [22], SSTAR [23], TB Profiler [24] and Mykrobe tested by us [25]. Mykrobe has several advantages in comparison with previous software:

1) it has an updated catalogue, increasing the sensitivity for determination of pyrazinamide resistance;

2) it allows the users to add their catalogues;

3) it has improved identification for non-tuberculosis mycobacterial species.

The Mykrobe software specificity and sensitivity were estimated in a previous study comparing phenotypic and whole-genome sequencing results obtained for 4362 isolates of *M. tuberculosis*. The estimated sensitivity of Mykrobe was 100, 95, 82, 99%, and the specificity is 99, 100 99, 99% respectively for rifampicin, isoniazid, pyrazinamide, and ethambutol [26]. Mykrobe software is not popular in Bulgaria and has not been applied. The present work aimed to determine the phenotypic and genotypic susceptibility of *M. tuberculosis* to antituberculosis drugs. Tasks of present work included determination of phenotypic susceptibility of *M. tuberculosis* strains isolated from Bulgarian patients, whole-genome sequencing of the strains and application of the software tool "Mykrobe" for drug-susceptibility testing.

Materials and Methods

This study includes 34 multi-resistant *M. tuberculosis* (MDR-TB) strains isolated from 34 Bulgarian patients' samples; out of them 9 were isolated in 2009, 23 — in 2010, and 2 strains were isolated in 2011. Their resistance has been phenotypically confirmed by BACTEC MGIT 960 System. The genotypic resistance for most of them was determined by the line probe assay (LPA) (71% of them were tested for first-line drugs and 21% were tested for second-line drugs) at the National Reference Laboratory of tuberculosis, NCIPD.

Isolation of M. tuberculosis strains from clinical samples

The studied clinical materials were processed by homogenization and decontamination, in accordance with the standard operating procedures described in the Methodological instructions for microbiological diagnosis and treatment of tuberculosis (Bulgarian Ministry of Health, 2009). Each sample was inoculated in two tubes Löwenstein–Jensen solid media and one tube with liquid media (Mycobacteria Growth Indicator Tube — MGIT). We used the products of "Becton Dickinson". The tubes were cultivated at 37°C. The result of cultivation on solid media was evaluated by the scale of semi-quantitative assessment of growth according to the above-mentioned guidelines.

MGIT liquid media tubes had a bar code and a fluorescence sensor on the bottom. The result was automatically generated by BACTEC MGIT 960 system and the Becton Dickinson's software. From the positive

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[1] World Health Organization, Geneva. WHO consolidated guidelines on tuberculosis. Module 4: treatment — drug-resistant tuberculosis treatment/ World Health Organization 2020. URL: https://apps.who.int/iris/bitstream/handle/10665/339991/9789289054966-rus.pdf

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World Health Organization (2013). Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children: policy update. World Health Organization. URL: https://apps.who.int/iris/handle/10665/112472

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test tubes we performed the immunochromatographic test BD MGIT TB Identification Test ("Becton Dickinson") in order to detect *Mycobacterium tuberculosis* complex. The Drug Sensitivity Tests (DST) was done using BACTEC and Geno Type MTBDR *plus* v.1.0 and Geno Type MTBDR *sl* v.1.0 ("Hain Lifescience").

Phenotypic DST of M. tuberculosis complex to first- and second-line anti-TB drugs

The phenotypic susceptibility of the strains to the following anti-tuberculosis drugs: streptomycin, isoniazid, rifampicin, ethambutol, ofloxacin, amikacin, kanamycin and capreomycin was evaluated by the proportion method using the fully automated BACTEC MGIT 960 system, according the manufacturer's instructions (BACTEC System User Guide MGIT 960, 2004). The tested critical concentrations were as follows: streptomycin — 1.0 μg/ml; isoniazid — 0.1 μg/ml; rifampicin — 1.0 μg/ml; ethambutol — 5.0 μg/ml; ofloxacin — 2.0 μg/ml; amikacin — 1.0 μg/ml; kanamycin — 5.0 μg/ml; capreomycin — 2.5 μg/ml.

Molecular genetic methods for detecting resistance to the first and second line anti-tuberculosis drugs

We used line probe assays (LPA) Geno Type MTBDR *plus* v.1.0 and Geno Type MTBDR *sl* v.1.0 ("Hain Lifescience") according the manufacturer's instructions. The tests were based on DNA-STRIP technology. Rifampicin resistance was found by detection of a mutation in the *rpoB* gene encoding the β-subunit of RNA polymerase. The resistance to isoniazid was searched in two genes: a mutation in the *katG* gene encoding peroxidase, which causes a high level of resistance, and a mutation in the *inhA* gene encoding enoyl-(acyl-protein carrier) reductase (NADH), causing a low level resistance. Resistance to the fluoroquinolones was detected by GenoType MTBDR sl, v.1.0, scoping for mutation in the *gyrA* gene encoding DNA gyrase. The resistance to aminoglycosides and cyclic peptides was detected by proved mutation in the *rrs* gene encoding the 16S rRNA.

DNA isolation

Phenotypically proven multidrug-resistant *M. tuberculosis* strains were grown on Löwenstein–Jensen medium for 35–42 days at 37°C. A full inoculation loop of fresh culture was resuspended in 400 μl TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.0)] in a 1.5–2.0 ml screw cap tube. The samples were incubated for 20 min at 80°C for inactivation of mycobacterial culture. DNA was isolated performing method described by van Soolingen and modified by us. [27]. In each tube to the lysozyme was added 1U RNase H followed by incubation for 4 h at 37°C than 70 μl of 10% sodium dodecyl sulfate (SDS) and 10 μl proteinase K at a concentration of 10 mg/ml were added and tubes were incubated for 24 h at 65°C. All other steps were performed following the original methodology. DNA quality was checked spectrophotometrically at optical density 1.8–1.9 OD measured on 260/280 nm.

Strain sequencing and bioinformatic analysis

Whole genomes of 34 *M. tuberculosis* resistant strains collected by the Bulgarian National Reference Laboratory of Tuberculosis were sequenced at the Supranational Reference Laboratory of Tuberculosis, San Raffaele Institute, Milan, Italy. Sequencing results were provided as FASTQ files written in fasta.qz format. Information about the whole genome of each strain was stored in two files: one with the amplicons ordered in 5 ' → 3 ' direction, and a second ordered in 3 ' → 5. The majority of the amplicons were 117 bp in size without adapter regions on both ends. Bacterial genome size was about 4.5 million base pairs. The size of each file containing genome data was about 250 MB. We installed Mykrobe software (www.mykrobe.com), version v.0.8.1 for desktop under Windows 10 [28] which is freely available for non-commercial use to analyze genome sequences. We loaded pair of readings sequenced in both directions. After overlaying of fragments on the catalogues used by Mykrobe offline, containing information about the genes determining drug resistance, drug resistance and related gene mutations were presented in a tabular form. The agreement between resistant phenotypic cultural method and Mykrobe software prediction tool was illustrated by Venn diagram[3] for each tuberculostatic. Reference vaccine strain BCG SL222 Sofia [29], originating from Russian vaccine strains BCG-I seed lot 374(a) was used as a negative reference control.

Results and Discussion

We determined the drug-susceptibility of 34 strains of *M. tuberculosis* collected at the National Reference Laboratory of Tuberculosis, NCIPD, Sofia, Bulgaria. All investigated strains were MDR, and in two of them, we proved extensive drug resistance (XDR). The phenotypic and genotypic drug resistance of strains is shown in **Table**.

Designations of strains are shown in the first column of Table. All phenotypically proven rifampicin and isoniazid-resistant strains were 100% confirmed by Mykrobe software. In thirty-one strains, rifampicin resistance was caused by S450L mutation — following the *M. tuberculosis* H37R nomenclature (or S531L according to the *Escherichia coli* nomenclature used by Hain Lifescience). One strain demonstrated H445Y mutation — according to *M. tuberculosis* H37Rv (H526), one was with H445D (H526D), and one with N432L (N513L) mutations in the *rpoB* gene.

S315T mutation responsible for isoniazid resistance was observed in the *katG* gene in strain N:22_09.

[3] URL: https://bioinformatics.psb.ugent.be/webtools/Venn/

Analysis of the drug susceptibility of 34 MDR *M. tuberculosis* strains by phenotypic identification with BACTEC system, line probe assay with Geno Type test and whole genome sequences with Mykrobe software

NRL CODE	BACTEC								Test «Geno Type»						«Mykrobe»									
	Streptomycin	Isoniazid	Rifampicin	Ethambutol	Ofloxacin	Amikacin	Kanamycin	Kapreomycin	Pyrazinamide (rpob mut)	Isoniazid (katg mut)	Isoniazid (inha mut)	Fluoroquinolones (gyramut)	Kanamycin/amikacin (rrsmut)	Ethambutol (embbmut)	Rifampicin	Isoniazid	Ethambutol	Streptomycin	Pyrazinamide	Ciprofloxacin	Fluoroquinolones	Ofloxacin	Moxifloxacin	Ciprofloxacin	Amikacin	Kanamycin
1_10	R	R	R	S	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A450X	P96L	S	S	S	S	S	S	S
6_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	A450X	P96L	S	S	S	S	S	S	S
7_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517X	L4S	S	S	S	S	S	S	S
8_09	R	R	R	S	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	S315T	S	S	S	S	S	S	S	S	S	S
8_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	P96L	S	S	S	S	S	S	S
10_10	S	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	S	S	S	S	S	S	S	S
11_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	S	S	S	S	S	S	S	S
12_10	R	R	R	S	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	P96L	S	S	S	S	S	S	S
13_10	S	R	R	R	S	S	S	S	H445Y(H526Y)	0	C-15T	NA	NA	NA	H445Y	C-15T	M306V	S	S	S	S	S	S	S	S	S
14_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	S	S	S	S	S	S	S	S
18_09	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	P96L	S	S	S	S	S	S	S
18_10	R	R	R	S	S	S	S	S	H445D(H526D)	0	C-15T	NA	NA	NA	H445D	C-15T	S	S	S	S	S	S	S	S	S	S
19_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	S	S	S	S	S	S	S	S
19_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	H82R	S	S	S	S	S	S	S
20_09	S	R	R	S	S	S	S	S	NA	NA	NA	NA	NA	NA	Q432L	C-15T	M306V	S	S	C1402X	S	S	S	S	S	S
20_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517 X	L4S	S	S	S	S	S	S	S
21_10	R	R	R	S	R	S	S	S	S450L(S531L)	0	C-15T	A90V	0	M306V	S450L(S531L)	C-15T	M306V	C517X	G97C	S	A90V	A90V	A90V	A90V	S	S
22_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	S315T	S	S	G105D	S	S	S	S	S	S	S
22_10	S	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	H82R	S	S	S	S	S	S	S

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End of Table.

NRL CODE	BACTEC								Test «Geno Type»						«Mykrobe»									
	Streptomycin	Isoniazid	Rifampicin	Ethambutol	Ofloxacin	Amikacin	Kanamycin	Kapreomycin	Pyrazinamide (rpob mut)	Isoniazid (katg mut)	Isoniazid (inha mut)	Fluoroquinolones (gyramut)	Kanamycin/amikacin (rrsmut)	Ethambutol (embbmut)	Rifampicin	Isoniazid	Ethambutol	Streptomycin	Pyrazinamide	Ciprofloxacin	Fluoroquinolones	Ofloxacin	Moxifloxacin	Ciprofloxacin	Amikacin	Kanamycin
23_10	NA	R	R	NA	R	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517X	S	S	A90V	A90V	A90V	A90V	S	S
24_10	R	R	R	R	R	S	S	S	S450L(S531L)	0	C-15T	A90V	0	M306V	S450L(S531L)	C-15T	M306V	C517X	S	S	A90V	A90V	A90V	A90V	S	S
34_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	S	S	S	S	S	S	S	S
37_09	R	R	R	R	S	S	S	S	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517X	G97C	S	S	S	S	S	S	S
38_10	NA	R	R	NA	R	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517X	V139A	S	D94H	D94H	D94H	D94H	S	S
39_10	R	R	R	S	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	S	S	S	S	S	S	S	S	S
41_10	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	0	0	M306V	S450L(S531L)	C-15T	M306V	A514X	S	S	S	S	S	S	S	S
45_10	R	R	R	S	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	S	S	S	S	S	S	S	S
49_10	NA	R	R	NA	S	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	S	S	S	S	S	S	S	S
52_10	R	R	R	S	R	S	S	S	S450L(S531L)	0	C-15T	NA	NA	NA	S450L(S531L)	C-15T	M306V	C517X	G97C	S	A90V	A90V	A90V	A90V	S	S
60_10	R	R	R	R	R	R	R	R	S450L(S531L)	0	C-15T	D94G	A1401G	M306V	S450L(S531L)	I194T	M306V	S	H82R	S	A90V	D94H	D94H	D94H	A-1400G	A-1400G
62_10	R	R	R	R	NA	NA	NA	NA	NA	NA	NA	D94G	A1401G	M306V	S450L(S531L)	C-15T	M306V	S	S	S	A90V	D94H	D94H	D94H	A-1400G	A-1400G
72_10	R	R	R	S	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	S450L(S531L)	C-15T	M306V	A514X	P69L	S	S	S	S	S	S	S
32_11	R	R	R	R	R	S	S	S	S450L(S531L)	0	C-15T	D94A	0	M306V	S450L(S531L)	C-15T	M306V	A514X	G97C	S	A90V	D94A	D94A	D94A	S	S
78_11	R	R	R	R	S	S	S	S	S450L(S531L)	0	C-15T	0	0	M306V	S450L(S531L)	C-15T	M306V	A514X	S	S	S	S	S	S	S	S
BCG SL222 Sofia	S	S	S	S	S	S	S	S	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S	S	S	S

Note. Results with BACTEC system-drug susceptible (S) and drug resistant (R). Geno Type detected mutations in *rpoB* gene conferring resistance to rifampicin are reported according to nomenclature in *M. tuberculosis H37Rv*. In brackets is the same mutation according to nomenclature in *E. coli*. Resistance conferring mutation to isoniazid is in the promoter region of *inhA* gene, a nucleotide substitution C-15T. Mutation in *rrs* gene leading to amikacin and kanamycin resistance is a nucleotide substitution A-1400G in rRNA gene. NA — no data. 0 — mutation not detected.

In strain, N:60_10 double mutation in the *inhA* gene was detected. I194T mutation in the *inhA* gene was proven in combination with C-15T mutation in the promoter region of the *inhA* gene. C-15T mutation in the promoter region of the *inhA* gene was found in 33 strains, which is responsible for isoniazid resistance. C-15T mutation is prevalent and most widespread (> 50%) among the MDR strains of *M. tuberculosis* isolated in Bulgaria with spoligotype SIT41 (TUR) [30]. These results were consistent with previous studies [30–33]. We can conclude that the C-15T mutation itself is a marker for isoniazid resistance in investigated strains with SIT41 spoligotype and is predictive for MDR.

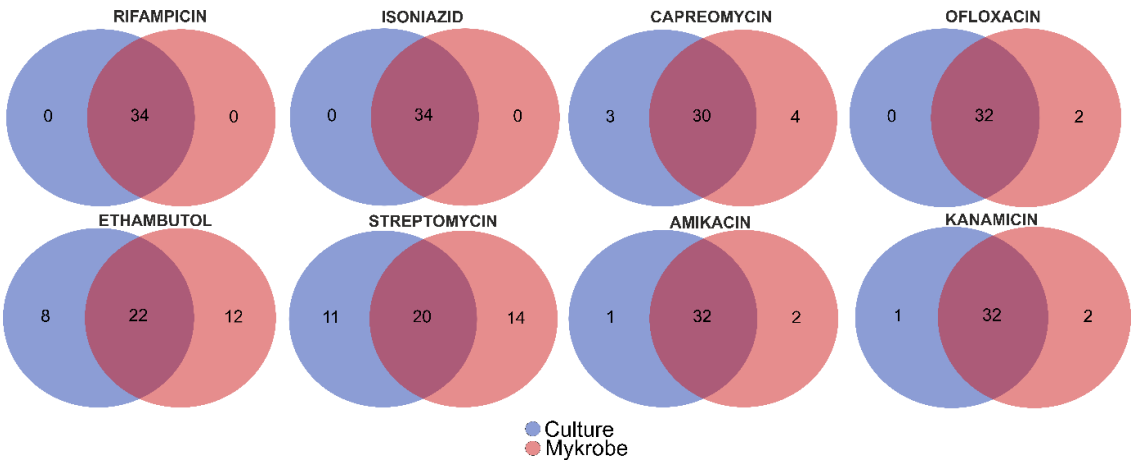
We observed a 75% (21/28) overlapping rate between BACTEC and Mykrobe for ethambutol resistance. In 4 strains (13%), M306V mutation in the *embB* gene was proven, which was expected to cause ethambutol resistance, but these strains were phenotypically susceptible. We can suggest that in these four strains M306V mutation is a polymorphism unrelated to phenotypic ethambutol resistance. Strain 22_09 was phenotypically identified as resistant, but mutation M306V or another related was not identified. This strain might have other, unknown mutations leading to ethambutol resistance

The sensitivity of some strains to streptomycin was very interesting. Phenotypically 87% (n = 27) of streptomycin-resistant strains were proven and only 59% (n = 19) were detected by Mykrobe. In 8 strains streptomycin sensitivity was detected by analysis of FASTQ files with Mykrobe, while BACTEC showed phenotypic resistance to this antituberculosis drug. For 19 strains proven by Mykrobe streptomycin resistance, we identified in 2 of them that streptomycin resistance was caused by A450X nucleotide mutation, in 9 by A514X, and in 8 by C517X nucleotide mutation in *rrs* gene encoding 16S rRNA which is responsible for streptomycin resistance. We can suggest that other unknown mutations, different from the described above in *rpsL*, *rrs*, and *gidB* genes, are responsible for streptomycin resistance. In other cases, streptomycin resistance can be caused by efflux or change in streptomycin targeting. Our investigation showed that the Mykrobe software tool has limited capability to test streptomycin resistance, giving a 30% error rate.

Different mechanisms of fluoroquinolone resistance were found in different strains [34]. In strains 21_10, 23_10, 24_10, 52_10, 60_10, and 62_10, resistance to ofloxacin, moxifloxacin and ciprofloxacin was caused by A90V mutation in *gyrA* gene and in strains 38_10, 60_10, 32_11 and 62_10, by D94H mutation in the same gene. Comparing phenotypic and genotypic resistance to ofloxacin, amikacin and kanamycin, we observed 100% coincidence of results. Six strains were phenotypically and genotypically proven as pre-XDR with 100% coincidence to fluoroquinolone resistance. A disadvantage of our study is the small number of proven MDR strains resistant to this drug. In the studied group of MDR strains, we observed two that were phenotypically and genotypically confirmed as XDR.

The susceptibility of the strains to pyrazinamide was evaluated only with Mykrobe. Seventeen (50%) pyrazinamide resistance strains were observed. Resistance was caused by six different mutations in the *pncA* gene. The most often detected mutation was P69L in 6 strains. G97C was the second most common mutation proven in 4 strains. Based on these results, we can conclude that resistance to pyrazinamide is a result of several mutations in the *pncA* gene.

All studied by us *M. tuberculosis* strains with Geno Type test and Mykrobe tool showed identical results for antituberculosis drug resistance. Mykrobe gave more full and detailed information (**Figure**). The disadvantage of Geno Type is that it does not cover all mu-



Venn diagram representing the agreement between resistant phenotypes identified by phenotypic cultural method and Mykrobe software prediction tool for 8 tuberculostatics.

Recently, new software tools were developed for the analysis of resistance of different species of microorganisms, which will be applied in our future studies [24, 35].

tations responsible for the resistance of *M. tuberculosis* to the currently applied anti-TB drugs.

Conclusion

Currently, different methods of identifying drug resistance have been developed and introduced into practice. Next-generation sequencing and bioinformatics data analysis are fast developing technologies and they will be used more widely soon. Next-generation sequencing technologies will be mandatory in the characterization and registration of new strains. Comparison of different methods showed that in some cases one could identify a mismatch between expected and observed phenotypes and genotypes. The genotypes include marker genes or other genome regions involved in drug resistance. Resistance can be caused by other unknown markers. This fact does not allow us to ignore phenotypic methods for the determination of antimicrobial resistance, and to prescribe drugs based on DNA analyses, despite enormous possibilities provided by whole-genome sequencing and bioinformatics. However, phenotypic methods do not determine the mechanism of resistance. Application of whole genome sequencing assay allows observing different genetic modifications associated with different mechanisms causing drug-resistance of *M. tuberculosis* complex. Different mutations cause different levels of resistance, and this will be the subject of our future investigations. Next-generation sequencing allows not only to compare data with phenotypically detected resistance but also to find relations between a mutation(s) and level of resistance.

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