

NEXT-GENERATION SEQUENCING OF DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES IN LOW-INCIDENCE COUNTRIES

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Abstract. Drug resistant tuberculosis (TB), especially multidrug (MDR) and extensively drug-resistant (XDR) TB, is still a serious problem in global TB control. Slovenia and North Macedonia are low-incidence countries with TB incidence rates of 5.4 and 10.6 in 2017, respectively. In both countries, the percentage of drug resistant TB is very low with sporadic cases of MDR-TB. However, global burden of drug-resistant TB continues to increase imposing huge impact on public health systems and strongly stimulating the detection of gene variants related with drug resistance in TB. Next-generation sequencing (NGS) can provide comprehensive analysis of gene variants linked to drug resistance in *Mycobacterium tuberculosis*. Therefore, the aim of our study was to examine the feasibility of a full-length gene analysis for the drug resistance related genes (*inhA*, *katG*, *rpoB*, *embB*) using Ion Torrent technology and to compare the NGS results with those obtained from conventional phenotypic drug susceptibility testing (DST) in TB isolates. Between 1996 and 2017, we retrospectively selected 56 TB strains from our National mycobacterial culture collection. Of those, 33 TB isolates from Slovenian patients were isolated from various clinical samples and subjected to phenotypic DST testing in Laboratory for Mycobacteria (University Clinic Golnik, Slovenia). The remaining 23 TB isolates were isolated from Macedonian patients and sent to our laboratory for assistance in phenotypic DST testing. TB strains included were either mono-, poly- or multidrug resistant. For control purposes, we also randomly selected five TB strains susceptible to first-line anti-TB drugs. High concordance between genetic (Ion Torrent technology) and standard phenotypic DST testing for isoniazid, rifampicin and ethambutol was observed, with percent of agreement of 77%, 93.4% and 93.3%, sensitivities of 68.2%, 100% and 100%, and specificities of 100%, 80% and 88.2%, respectively. In conclusion, the genotypic DST using Ion Torrent semiconductor NGS successfully predicted drug resistance with significant shortening of time needed to obtain the resistance profiles from several weeks to just a few days.

Key words: tuberculosis, *Mycobacterium tuberculosis*, drug resistant tuberculosis, multidrug resistant tuberculosis, next-generation sequencing, Ion Torrent, phenotypic drug susceptibility testing.

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СЕКВЕНИРОВАНИЕ СЛЕДУЮЩЕГО ПОКОЛЕНИЯ ЛЕКАРСТВЕННОУСТОЙЧИВЫХ КЛИНИЧЕСКИХ ИЗОЛЯТОВ *Mycobacterium tuberculosis* В СТРАНАХ С НИЗКИМ УРОВНЕМ ЗАБОЛЕВАЕМОСТИ

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Резюме. Лекарственноустойчивый туберкулез (ЛУ-ТБ), особенно ТБ с множественной лекарственной устойчивостью (МЛУ-ТБ) и ТБ с широкой лекарственной устойчивостью (ШЛУ-ТБ), до сих пор представляет серьезную проблему глобального контроля ЛУ-ТБ. Словения и Северная Македония являются странами с низким уровнем заболеваемости ЛУ-ТБ, которые в 2017 г. составили 5,4 и 10,6 на 100 тыс. населения, соответственно. В обеих странах доля ЛУ-ТБ крайне низка, выявляются лишь спорадические случаи МЛУ-ТБ. Однако глобальный уровень ЛУ-ТБ продолжает расти, оказывая существенное влияние на систему здравоохранения, а также способствуя обнаружению генетических вариантов, связанных с ЛУ-ТБ. Секвенирование следующего поколения (next generation sequencing [NGS]) может предоставить возможность глубокого анализа генетических вариаций, ассоциированных с лекарственной устойчивостью *Mycobacterium tuberculosis*. Целью настоящего исследования была оценка применимости анализа таких генов (*inhA*, *katG*, *rpoB*, *embB*) с использованием технологии Ion Torrent и сравнения данных NGS и стандартного фенотипического определения лекарственной чувствительности (drug susceptibility testing [DST]) изолятов ЛУ-ТБ. Было проведено ретроспективное исследование 56 ЛУ-ТБ штаммов, полученных в течение 1996–2017 гг. из Национальной коллекции культур микобактерий (Голник, Словения). Из них, 33 изолята было получено от пациентов из Словении из различных клинических образцов, подвергнутых фенотипическому тестированию DST в Лаборатории микобактерий (University Clinic Golnik, Slovenia). Остальные 23 изолята были выделены от пациентов из Северной Македонии, и переданы в нашу лабораторию для помощи в проведении тестирования DST. ЛУ-ТБ штаммы обладали моно-, поли- или множественной лекарственной устойчивостью. В качестве контрольных образцов были использованы случайным образом отобранные пять штаммов, чувствительных к противотуберкулезным препаратам первого ряда. В результате, показана высокая степень соответствия между генетическими (Ion Torrent) данными и результатами стандартного фенотипического DST для изониазида, рифампицина и этамбутола, с показателем согласия 77, 93,4 и 93,3%, чувствительностью — 68,2, 100 и 100%, специфичностью — 100, 80 и 88,2% соответственно. Таким образом, генотипическое тестирование лекарственной устойчивости с применением NGS в формате полупроводниковой технологии Ion Torrent успешно выявило лекарственную устойчивость с резким сокращением времени, необходимого для получения профиля резистентности — с нескольких недель до двух дней.

Ключевые слова: туберкулез, *Mycobacterium tuberculosis*, лекарственноустойчивый туберкулез, туберкулез с множественной лекарственной устойчивостью, секвенирование следующего поколения, Ion Torrent, фенотипическое определение лекарственной чувствительности.

Introduction

Mycobacterium tuberculosis (MT), an obligate pathogen that causes tuberculosis (TB), is highly transmissible agent with significant morbidity and mortality. Global strategies to treat and control TB are designed to accurately and rapidly diagnose, treat and reduce the transmission of TB. The increasing burden of multidrug resistant (MDR) and extensively drug-resistant (XDR) TB is a serious problem in global TB control. Slovenia and North Macedonia are countries with low incidence of TB cases with incidence rates 5.4 and 10.6 in 2017, respectively [1]. Moreover, in Slovenia in the period between 1996 and 2017 the average percentage of any drug resistance against isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM) was 4.13%, dropping from 6.55% in 1996 to 1.89% in 2017. In the same period, 25 sporadic MDR-TB cases were observed with the last two cases

of MDR-TB noted in 2009 and in 2017. For North Macedonia, the average percentage of any drug resistance against INH, RIF, EMB and SM (they do not perform DST testing for PZA) was higher and accounted for 10.84%, dropping from 9.93% in 2001 to 5.81% in 2017. During 1996 and 2017 75 MDR-TB cases were observed in North Macedonia with MDR-TB cases appearing each year [2,3,4].

Currently, the reference method for determining drug resistance in clinical laboratory is culture based drug susceptibility testing (DST), using either solid or liquid media. However, this method is labour-intensive, time-consuming (due to slow growth of MT it takes weeks to months to obtain DST results), technically challenging and requires handling viable and potentially infectious cultures of MT bacilli [1].

As alternative to phenotypic DST, several commercially available molecular assays rapidly detect common mutations related to resistance to isoniazid, rifampicin and some second-line anti-TB

drugs. GeneXpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, USA) is a real-time PCR based assay that detects resistance directly from sputum samples. On the other hand, there are several line probe assays recommended by WHO, including GenoType MTBDRplus and MTBDRsl (Hain Lifescience, Nehren, Germany) and Nipro NTM + MDRTB II (Nipro Corporation, Osaka, Japan). However, the mechanisms of drug resistance are complex and not completely understood. Therefore, one of the main limitations of such molecular tests is that they evaluate only limited number of mutations linked with drug resistance in TB [9].

In recent years, significant and continued progress in next-generation sequencing (NGS) made this technology a promising clinical tool in comprehensive analysis of gene variants linked to drug resistance in TB [9]. Besides known mutations, NGS facilitates the discovery of novel variants in the entire coding regions of several genes previously implicated in MDR and/or XDR-TB resistance [9].

In this study, we examined the feasibility of a full-length gene analysis for the drug resistance related genes using Ion Torrent technology and compared the results with those obtained from conventional phenotypic drug susceptibility testing (DST) in 61

TB isolates. In this short paper, we compare molecular results with phenotypic DST results for isoniazid (INH), rifampicin (RIF) and ethambutol (EMB), anti-TB drugs used in first-line treatment regimens.

Materials and methods

TB strains. Between 1996 and 2017, we retrospectively selected 33 TB strains that were isolated from various clinical samples of Slovenian patients and subjected to phenotypic DST testing according to routine procedures in Laboratory for Mycobacteria (University Clinic Golnik, Slovenia). Between years 1999 and 2010, National Laboratory for Mycobacteria (Institute for Pulmonary Diseases and Tuberculosis Skopje, North Macedonia) sent to our laboratory 23 TB strains for assistance in phenotypic DST testing. TB strains included were either mono-, poly- or MDR resistant. For control purposes, we also randomly selected five TB strains susceptible to first-line anti-TB drugs. Detailed information about the type of resistance is presented in Table 1.

Phenotypic drug susceptibility (DST) testing. Phenotypic drug resistance to first-line drugs was determined using Bactec MGIT 960 System (BD) from pure culture of MT strains. Critical concentrations

Table 1. Distribution of *Mycobacterium tuberculosis* (MT) strains included in the study according to resistance profile and country of origin

Resistance profile	Country of origin (survey period)		Number of TB patients
	Slovenia (1996–2017)	North Macedonia* (1999–2010)	
Sensitive	5	0	5
Mono-resistance	11	0	11
INH	2	0	2
RIF	3	0	3
PZA	6	0	6
Poly-resistance	6	1	7
PZA+SM	3	0	3
INH+PZA+SM	1	0	1
INH+PZA	2	0	2
INH+EMB+PZA+SM+FLQ	0	1	1
MDR	16	22	38
INH+RIF	1	7	8
INH+RIF+SM	1	0	1
INH+RIF+EMB	0	1	1
INH+RIF+PZA	0	3	3
INH+RIF+EMB+PZA	1	1	2
INH+RIF+EMB+SM	2	3	5
INH+RIF+PZA+SM	1	0	1
INH+RIF+EMB+PZA+SM	8	6	14
INH+RIF+EMB+PZA+SM+FLQ	2	1	3
Total	38	23	61

Notes. INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol; PZA: Pyrazinamide; SM: Streptomycin; FLQ: fluoroquinolones (either ofloxacin, ciprofloxacin or moxifloxacin); MDR: multi-drug resistance; resistance to (at least) INH and RIF; *National Laboratory for Mycobacteria (Institute for Pulmonary Diseases and Tuberculosis Skopje, North Macedonia) sent 23 TB strains for assistance in phenotypic DST testing.

Table 2. Comparison of NGS and phenotypic (BACTEC MGIT 960) drug susceptibility testing (DST) results for *Mycobacterium tuberculosis* (MT) isolates (N = 61)

NGS	Phenotypic DST		% of agreement	Sensitivity (%), 95% CI	Specificity (%), 95% CI	PPV (%), 95% CI	NPV (%), 95% CI
	Resistant	Susceptible					
Isoniazid* (N = 61)							
mutated	30	0	77.0	68.2 (72.3–94.8)	100.0 (80.5–100.0)	100.0 (90.8–100.0)	54.8 (51.6–89.8)
non-mutated	14	17					
Rifampicin (N = 61)							
mutated	41	4	93.4	100.0 (90.1–100.0)	80.0 (56.3–94.3)	91.1 (78.8–97.5)	100.0 (79.4–100.0)
non-mutated	0	16					
Ethambutol (N = 60**)							
mutated	26	4	93.3	100.0 (86.8–100.0)	88.2 (72.6–96.7)	86.7 (69.3–96.2)	100.0 (88.4–100.0)
non-mutated	0	30					

Notes. *Mutations in *inhA* and *katG* included in the comparison; **one TB isolate was not tested for ethambutol and was therefore excluded in further comparison; N: number of isolates included; NGS: next-generation sequencing (Ion Torrent, Ion AmpliSeq TB Research Panel, Thermo Fisher Scientific); 95% CI: 95% confidence interval; PPV: positive predictive value; NPV: negative predictive value

for anti-TB drugs tested were as follows: INH 0.1 µl/ml (high level 0.4 µl/ml), RIF 1 µl/ml and EMB 5 µl/ml (high level 7.5 µl/ml).

DNA extraction. Mycobacterial genomic DNA was isolated from pure cultures of *M. tuberculosis* using previously described protocol [13]. Purified genomic DNA was stored at -20 °C in Slovenian National Mycobacterial DNA Collection until further analysis.

AmpliSeq Library preparation, sequencing, data analysis and interpretation. Nucleic acid quality and quantity were assessed using NanoDrop 2000 (Thermo Scientific) 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 with drug resistance in genomic DNA extracted from MT isolates, AmpliSeq libraries were generated using the AmpliSeq™ Kit for Chef DL8 and the Ion AmpliSeq TB Research Panel. This panel amplifies 109 amplicons in two highly multiplexed PCR reactions covering coding sequences of eight genes related to drug resistance (including *inhA*, *katG*, *rpoB*, and *embB*). 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. 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 caller analysis were performed with the Torrent Suite software version 5.6 (all reagents, instruments and software Thermo Fisher Scientific). The sequencing data were analysed manually, comparing the determined variants with published data and data available in the Tuberculosis Drug Resistance Database [7]. Sequence of MTB H37Rv (NC_000962.3) was used as the reference sequence. The resistance genotyping profiles obtained with manual approach were compared to the results of phenotypic DST testing. Sequence data are available in SRA NCBI database under BioProject accession number PRJNA551916.

Statistics. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for NGS were calculated using GraphPad Prism v 6.04 (GraphPad Software, Inc., CA, USA).

Results and discussion

The NGS analysis was successfully used to predict drug resistance profiles. Percentage of agreement between both methods and corresponding sensitivities, specificities, positive predictive values and negative predictive values are presented in Table 2.

The percent of agreement between molecular and phenotypic DST testing in our study was the lowest for INH and accounted for 77% with sensitivity of 68.2% and specificity of 100%. The studies based

on susceptibility testing so far have not demonstrated complete agreement between the phenotypic and genotypic assays [6, 7, 12, 14, 16]. Hazbon и соавт. [6] reported in their study on a large number of TB isolates that around 34% of phenotypically resistant isolates are not associated with any genotypic mutations in genes most commonly linked to INH resistance (*katG*, *inhA*, *kasA*, *ahpC* and *ndh*), suggesting that many genetic causes of INH resistance are yet to be discovered. Supporting this data, recent systematic review [12] on INH resistance demonstrated that 84% of global phenotypic INH resistance is associated with mutations in *katG*, *inhA* and *ahpC-oxvR* intergenic region, while the other 16% are, probably, linked to genetic changes in other genes.

Our study indicates high percentage of agreement between both methods used for RIF resistance testing (93.4%) with high sensitivity (100%) and specificity (80%). This is concordant with published literature [5, 10, 13] which reported very good agreement between genotypic and phenotypic DST. Zaw et al. [15] showed that mutations in *rpoB* gene (specifically mutations within 81-bp RIF-resistance determining region; RRDR) are responsible for approximately 95% of all RIF resistance cases in TB strains.

Similarly, we also noticed high percent of agreement between phenotypic and genotypic DST results for EMB (93.3%) with high sensitivity (100%) and specificity (88.2%). These results are concordant with some of the published studies [9, 10], which showed large overlap in the estimated prevalence of EMB resistance by genetic sequencing and the estimated prevalence by phenotypic testing. However, several other studies observed discrepancies between the presence of common mutations at codon 306 of *embB* gene and phenotypic EMB resistance [5,

8]. Authors of these studies determined mutations in *embB306* also in clinical *M. tuberculosis* isolates that are susceptible to EMB. One large study (with international TB isolates included) noted that almost half of the TB isolates with *embB306* mutations were fully susceptible to EMB. Furthermore, they found strong correlation between *embB306* mutations and resistance to any antibiotic, suggesting that *embB306* mutations were responsible for broad antibiotic resistance [5]. In our study with relatively low number of patients included, we observed 20 (20/60; 33.3%) TB isolates with *embB306* mutations. Of those 20 MT isolates that harboured *embB306* mutations, four (4/20; 20.0%) TB isolates that were phenotypically susceptible to EMB, remaining 16 (16/20; 80.0%) TB isolates were phenotypically resistant to EMB.

Overall, this study describes the first utilization of Ion Torrent sequencing of full-length genes to characterize drug resistant MT isolates for In conclusion, genotypic DST using Ion Torrent semiconductor NGS has the potential to provide useful information several weeks before phenotypic DST results. Therefore, genetic sequencing (NGS) seems to be a valuable tool for surveillance of drug resistance in TB. Before this takes place, there is a need to standardise the whole procedure including DNA extraction, recording, and reporting and data interpretation.

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