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Original Article

Whole Genome Sequencing of Bulgarian Rifampicin Resistant *Mycobacterium tuberculosis* Strains

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Abstract

Introduction: The transmission of drug-resistant tuberculosis is one of the greatest challenges facing the global tuberculosis control.

Aim: The aim of the study was to investigate the resent transmission of rifampicin resistant tuberculosis in Bulgaria and to describe the mutations related to the antimicrobials' resistance using whole genome sequencing.

Materials and methods: As part of an ECDC funded pilot study for evaluation of the systematic use of whole genome sequencing (WGS) of *Mycobacterium tuberculosis* (MTB) surveillance (EUSeqMyTB), Bulgaria provided 65 rifampicin resistant isolates over a three years' timeframe (2017-2019) representing 87.5% of the notified rifampicin resistant cases. Drug resistance prediction and relatedness analysis of the resistant isolates was performed in collaboration with San Raffaele Scientific Institute, Milan, Italy.

Results: Almost all of the isolates were identified as Euro-American lineage (96.9%); 18.5% of the isolates were found to be resistant to fluoroquinolones, but no mutations conferring resistance to bedaquiline or linezolid could be identified. Less than half (43.3%) of the isolates were clustered (<5 SNPs distance) into a total of seven national SNP-based clusters, while a total of six isolates were found to be part of different cross-border clusters. All clustered cases originated from Bulgaria.

Conclusions: WGS has proven to be a reliable tool for surveillance and tracing of recent transmission of tuberculosis and has the potential for resistance prediction for most of the antituberculosis drugs.

Keywords

multidrug resistance, transmission

Abbreviations:

ECDC: European Centre for Disease Prevention and Control

INTRODUCTION

Transmission of drug-resistant tuberculosis (TB) is one of the greatest challenges in the TB control. Globally in 2019, almost half a million individuals had rifampicin or multidrug-resistant tuberculosis (RR/MDR-TB) (3.3% of the new TB cases and 17.7% of previously treated TB cases).[1]

Although the TB incidence tend to decline in the recent years, Bulgaria remains a high priority country with notification rate of 19.1 per 100 000 in 2019, which is twice as high as that of the European Union/European Economic Area (EU/EEA) - 9.6 per 100 000.[2] The bacteriologically confirmed RR/MDR-TB cases in EU/EEA for 2019 were 834 (3.4%) and 11 of them were reported from Bulgaria, which was 2.4% of all the cases with drug susceptibility testing (DST) result in the country.[2] Treatment of such patients is long (about 24 months) and expensive, a successful treatment outcome being hard to achieve – barely 45.7%.[2]

Despite the implementation of reliable PCR techniques in the primary TB diagnosis settings worldwide, whole genome sequencing (WGS) stands out as the most sensitive typing method to trace transmission chains providing simultaneously drug resistance prediction to most of anti-tuberculous compounds.

In an ECDC funded pilot study for evaluation of WGS systematic use for *M. tuberculosis* surveillance (EUSeqMyTB), about 75% of the rifampicin resistant *M. tuberculosis* isolates from 28 EU/EEA were sequenced in a three-year term (2017-2019).[3] The National Reference Laboratory of tuberculosis (NRL TB), Sofia, Bulgaria confirmed rifampicin or multidrug resistance in 65 strains of 60 TB patients for the three-year period from 2017 to 2019, collected from all over the country.

AIM

The aim of the study was to reveal the resent transmission of rifampicin-resistant tuberculosis in Bulgaria and to describe the mutations related to antimicrobials' resistance using whole genome sequencing.

MATERIALS AND METHODS

The isolate's selection criteria was any *M. tuberculosis* strain with resistance to rifampicin (i.e. RR-TB or MDR-TB) based on genotypic and phenotypic drug susceptibility testing, provided by the Bulgarian TB laboratory network and confirmed in NRL TB. The study period was from 2017 to 2019. In the summarized results the multiple cultures from the same patient were excluded from the relatedness analysis so that each patient was represented by a single isolate. The isolates were twice pseudo anonymized by unique codes and no identifiable personal data was gathered.

The drug susceptibility testing was performed in NRL TB Sofia by BACTEC MGIT 960 system for antituberculosis drugs as follows: rifampicin, isoniazid, streptomycin, ethambutol, ofloxacin, moxifloxacin, amikacin, kanamycin, capreomycin and linezolid to the current critical concentrations.[4,5] The Line Probe Assay Genotype MTBDR *plus/sl* was used to detect the most common mutations associated with the resistance to the main first and second line antituberculosis drugs.[6,7] In order to summarize the data, we used descriptive statistical analysis.

WGS and data analysis were done at the San Raffaele Scientific Institute, Milan, Italy. For WGS-based relatedness analysis, there were performed core genome multilocus sequence typing (cgMLST) and the single nucleotide polymorphism (SNP)-based approach using the MTBseq pipeline.[8] The minimum spanning tree was calculated using Grapetree.[9] A cluster was defined as two or more isolates having a difference ≤5 SNPs.

RESULTS

For the period from 2017 to 2019, rifampicin or multidrug resistance was found in 65 strains of 60 Bulgarian TB patients. The coverage of the RR/MDR-TB strains, which were examined in Bulgaria, was estimated to be 87.5% according to ECDC notification data.[3] All rifampicin resistant TB cases were affected by pulmonary tuberculosis. The majority were males (n=41, 68.3%) with mean age 45.3 (range: 19 to 77 years). Most of the males were previously treated for tuberculosis (n=22) versus 19 new cases. Females were 19 (31.7%), with mean age 39.6 (range 23-71). Most of the females were new cases (n=14), versus 5 cases with previous treatment history.

The distribution of the RR/MDR-TB cases in the country for the three-year period is shown in **Fig. 1**. The most affected districts were Plovdiv (n=9), Montana (n=6), and Pernik (n=5).

Figure 1. Distribution of the RR/MDR-TB cases (2017-2019) in Bulgaria

Most of the Bulgarian isolates were identified as Euro-American lineage (96.9%) and only 3.07% were Beijing (n=2). The relatedness analysis allowed identification of 26 patients (43.3%) as part of seven clusters **(Fig. 2, Table 1)**. The size of the clusters varied from two to six patients. There were no cases of foreign origin involved in a cluster.

The transmission link for cluster A and one of the Montana's clusters – B, was the place of residence.

Cluster C contained family members with different households and addresses.

The northeastern cluster D had two patients with the same place of residence and two previously treated indi-

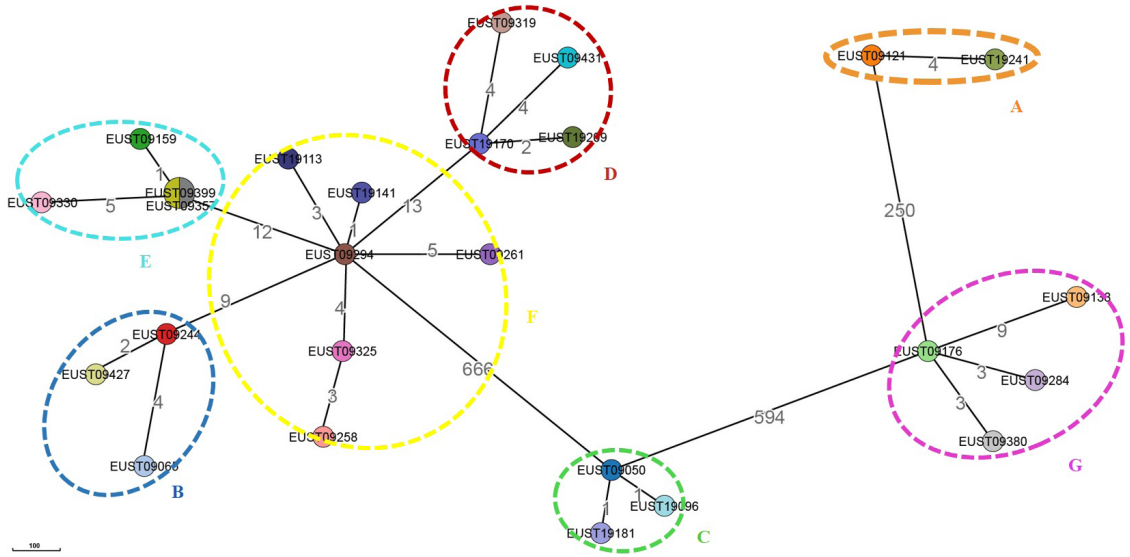

Figure 2. Minimum spanning tree of 26 Bulgarian RR-TB isolates. The numbers on the branches indicate the genetic distance in SNPs differences to the nearest isolate. SNP differences between distant strains cannot be reconstituted by summing the number of SNPs on the branches. SNP: single-nucleotide polymorphism.

Table 1. Description of the national clusters identified in the study

Cluster	Cases (n)	District	Drug resistance	Lineage
A	2	Pernik n=2	RIF-R (*rpoB*: Ser450Trp; n=2)	4.8; mainly T
B	3	Montana n=3	RIF-R (*rpoB*: Ser450Leu; n=3) INH-R (*inhA* prom.: C-15T n=3) STR-R (*rrs*: A514C; n=3) EMB-R (*embB*: Met306Val; n=3) PZA-R (*pncA*:Pro69Leu; n=3)	4.2.2.1; TUR
C	3	Varna n=1 Haskovo n=1 Targovishte n=1	RIF-R (*rpoB*: His445Asp; n=3) INH-R (*katG*: Ser315Thr; n=3)	4.4.1.1; S-type
D	4	Varna n=2 Dobrich n=1 Gabrovo n=1	RIF-R (*rpoB*: Ser450Leu; n=4) INH-R (*inhA* prom.: C-15T; n=4) STR-R (*rrs*: snp C517T; n=4) EMB-R (*embB*: Met306Val; n=4) FQ-R (*gyrA*: Ala90Val; n=4)	4.2.2.1; TUR
E	4	Montana n=3 Sofia province n=1	RIF-R (*rpoB*: Ser450Leu; n=4) INH-R (*inhA* prom.: C-15T; n=4) STR-R (*rrs*: snp A514C; n=4) EMB-R (*embB*: Met306Val; n=4) FQ-R (*gyrA*: Ala90Val; n=1)	4.2.2.1; TUR

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F	6	Dobrich n=2 Plovdiv n=1 Gabrovo n=1 Sofia n=1 Razgrad n=1	RIF-R (*rpoB*: Ser450Leu; n=6) INH-R (*inhA* prom.: C-15T; n=6) EMB-R (*embB*: Met306Val; n=4) PZA-R (*pncA*: His82Arg; n=5) FQ-R (*gyrA*: Ala90Val; n=1) FQ-R (*gyrA*: Ala90Val + *gyrB*Ala504Val; n=1)	4.2.2.1; TUR
G	4	Plovdiv n=2 Silistra n=1 Gabrovo n=1	RIF-R (*rpoB*: Ser450Leu; n=4) INH-R (*katG*: Ser315Thr; n=4) EMB-R (*embB*: Met306Val; n=4) PZA-R (*pncA*: ins2288846 A C; n=4) FQ-R (*gyrA*: Asp94Gly; n=1) AMK, KAN, CAP(*rrs*: A1401G; n=3)	4.8; mainly T

RIF: rifampicin; INH: isoniazid; STR: streptomycin; EMB: ethambutol; PZA: pyrazinamide; FQ: fluoroquinolones; AMK: amikacin; KAN: kanamycin; CAP: capreomycin; R: resistant

viduals where the source of infection and the missing link with the remote Gabrovo's case could be beyond the studied three-year period of time.

The second cluster of the Montana district (E) contained only new cases – evidence for ongoing transmission of MDR-TB. The epidemiological connection with the patient from Sofia province was not determined, but the two districts are near to one another.

Cluster F was formed from patients with various places of residence and half of them were previously treated. Two relatives were found in this group.

The Plovdiv cluster G was from previously treated individuals, originated from the town of Plovdiv and new remote patients with no clarified connection with the main cluster.

Out of sixty cases, eight (13.3%) were monoresistant to rifampicin, 86.7% were multidrug resistant tuberculosis. Resistance to fluoroquinolones (preXDR-TB) was found in 18.5% (n=12) of the isolates. The MDR-TB strains with resistance to fluoroquinolones and injectable second line drugs meeting the definition for XDR-TB (before the update in 2021) were three (5%) **(Fig. 3)**. There was no detected resistance to bedaquiline or linezolid so far.

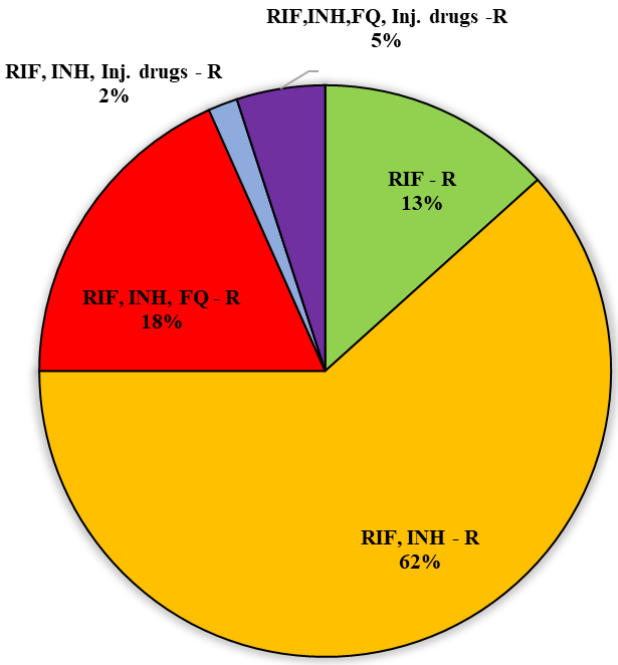

Figure 3. Resistance to antituberculosis drugs of the RR/MDR-TB isolates. RIF: rifampicin; INH: isoniazid; FQ: fluoroquinolones; Inj. drugs: at least one of the second line injectable drugs (amikacin, kanamycin, and capreomycin); R: resistant.

WGS data provided comprehensive information about the resistance to the majority of the antituberculosis drugs: rifampicin, isoniazid, ethambutol, pyrazinamide, fluoroquinolones, linezolid, bedaquiline, and the injectable second-line drugs **(Table 2)**.

The most commonly detected mutations in the Bulgarian RR/MDR-TB strains were Ser450Leu in the *rpoB* gene, C-15T mutation in the *inhA* promoter region or Ser315Thr in *katG* gene, Met306Val in *embB*, and Ala90Val in *gyrA* gene. The resistance to pyrazinamide and streptomycin was

Table 2. List of analyzed genomic region, results for the Bulgarian RR/MDR-TB strains and comparison with phenotypic DST results by BACTEC 960 MGIT

Anti TB Drug	Genomic region	Gene	Detected mutation by WGS, n	pDST
Rifampicin	Rv0667	*rpoB*	Ser450Leu (tcg/tTg), n=47	R n= 47
			His445Asp (cac/Gac), n=5	R n=5
			Asp435Val (gac/gTc), n=2	R n=2
			Ser450Trp (tcg/tGg), n=2	R n=2
			Leu430Pro (ctg/cCg), n=1	R n=1
			Ser441Leu (tcg/tTg), n=1	R n=1
			Gln432Pro (caa/cCa), n=1	R n=1
			Val170Phe (gtc/Ttc), n=1	R n=1
Isoniazid	Rv1483	*inhA promoter region*	C-15T (atc/aCc), n=26	R n= 26
	Rv1908c	*katG*	Ser315Thr (agc/aCc), n=18	R n= 18
			Ser315Asn (agc/aAc), n=1	R n=1
			Co-occurrence inhA+katG mutation, n=3	R n=3
	Rv2428	*ahpC*	C-57T, n=1	R n=1
			Not found	R n=2; S n=9
Ethambutol	Rv3795	*embB*	Met306Val (atg/Gtg), n=25	R n=24; S n=1
			Met306Ile (atg/atA), n=8	R n=3; S n=5
			Ala454Thr (gcg/Acg), n=2	S n=2
			Gly406Asp (ggc/gAc), n=2	S n=2
			Ser297Ala (tcg/Gcg), n=2	R n=1; S n=1
			Gln497Lys (cag/Aag), n=1	S n=1
			snp 4243225 C A, n=1	S n=1
Pyrazinamide	Rv2043c	*pncA*	Pro69Leu (cca/cTa), n=3 His82Arg (cat/cGt), n=6 Leu4Ser (ttg/tCg), n=2 His137Pro (cat/cCt), n=1 Thr76Pro (act/Cct), n=1 Gly105Asp (ggc/gAc), n=1 ins 2288846 A C, n=4 ins 2288708 G C, n=1	Not performed
Fluoroquinolones	Rv0006	*gyrA*	Ala90Val (gcg/gTg), n=8	R n=1; S n=7
			Asp94Gly (gac/gGc), n=2	R n=2
			Gly88Ala (ggc/gCc), n=1	S n=1
	Rv0005	*gyrB*	Not found	
			co-occurrence *gyrA* Ala90Val (gcg/gTg)+*gyrB* Ala 504 Val (gcg/gTg), n=1	R n=1
Amikacin, kanamycin, capreomycin	Rvnr01	*rrs*	A1401G, n=3	R n=3
Kanamycin	Rv2416c	*eis*	Not found	
Capreomycin	Rv1694	*tlyA*	Not found	

Streptomycin	Rv0682	*rpsL*	Lys43Arg (aag/aGg), n= 2 Lys88Arg (aag/aGg), n= 2	R n=2 R n=2
	Rvnr01	*rrs*	C517T, n=5 A514C, n=8	R n=5 R n=8
Bedaquiline	Rv0678	*mmpR*	Not found	Not performed
	Rv1305	*atpE*	Not found	

R: resistant; S: susceptible; n: number

coded by various mutations. The genotype-phenotype correlation was the strongest for rifampicin and the injectable drugs (amikacin, kanamycin, capreomycin, streptomycin), and significantly weak for ethambutol.

DISCUSSION

The Beijing lineage is globally distributed due to its high virulence and transmissibility. It is most common in China and Mongolia (more than 80%)[10] and is associated with MDR-TB outbreaks all over the world, including in high burden countries like Ukraine[11]. Although the Beijing lineage keeps the second position of distribution in EU/EEA (about 30% of the sequenced strains) in Bulgaria, it represents only 3% amongst the MDR-TB cases.[3] The trend of scarce spreading remains steady with the years (3.2% for the period of 2007-2011)[12] and could be explained with the small numbers of imported TB cases in the country.

The results of GenoType MTBDR *plus/sl* and WGS analysis of the genetic regions related to resistance showed equal results for rifampicin, isoniazid, fluoroquinolones, and the injectable second line drugs. However, the prediction for bedaquiline or linezolid resistance by molecular method has been available only by WGS so far. The drug resistance pattern of the clinical isolates in general remains the same in the years with the distinctive mutation C-15T in the *inhA* promoter region, related to low-level isoniazid resistance.[13]

WGS analysis failed in resistance prediction for two phenotypically isoniazid-resistant Bulgarian strains, probably because of mutation outside the analyzed genomic regions and other factors contributing to the resistance to this drug.[3,14,15]

The phenotypic DST for ethambutol is considered not reliable and reproducible[16] and the contribution of each mutation to drug resistance is difficult to evaluate.

Since the testing of ofloxacin is not recommended due to dropping out of resistant-TB treatment regimens[17] and the cross-resistance levels with the other representatives of the fluoroquinolones are not utter, the results of phenotypic DST is not represented in the table above.

Since January 2021, the definition of XDR-TB has been updated[18] and already covers the resistance to rifampicin, isoniazid, fluoroquinolone(s), and bedaquiline/linezolid. There were no detected MDR-TB strains with such pattern of resistance among the sequenced Bulgarian samples in the conducted study, but a few were found in Italy.[19] Nevertheless, the considerable number of the pre-XDR-TB isolates in Bulgaria (18.5%) remains a concern.

CONCLUSIONS

The first detailed characterization of rifampicin resistant strains isolated on the territory of Bulgaria by WGS was performed. The conducted study highlighted the sustainable trend of RR/MDR-TB transmission within the country of strains with well-known features. So far, the Beijing lineage has not affected the dynamics of tuberculosis in Bulgaria. WGS has proven to be a reliable tool for surveillance but in order to assess the transmission dynamics, it cannot be used without additional epidemiological data. The method has the potential for resistance prediction after adaptation to the diagnostic requirements – sequencing from specimen, simplifying the procedure and lowering the costs.

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Полногеномное секвенирование болгарских штаммов *Mycobacterium tuberculosis*, устойчивых к рифампицину

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Резюме

Введение: Передача лекарственно-устойчивого туберкулёза является одной из самых серьёзных проблем, стоящих перед глобальной борьбой с туберкулёзом.

Цель: Цель исследования состояла в том, чтобы изучить недавнюю передачу устойчивого к рифампицину туберкулёза в Болгарии и описать мутации, связанные с устойчивостью к противомикробным препаратам, с использованием полногеномного секвенирования.

Материалы и методы: В рамках финансируемого Европейским центром превенции и контроля инфекционных заболеваний пилотного исследования по оценке систематического использования полногеномного секвенирования (ПГС) надзора за *Mycobacterium tuberculosis* (MTB) (EUSeqMyTB) Болгария предоставила 65 устойчивых к рифампицину изолятов за трёхлетний период (2017 – 2019 гг.), что составляет 87.5% зарегистрированных случаев устойчивости к рифампицину. Прогнозирование лекарственной устойчивости и анализ родства устойчивых изолятов выполнялись в сотрудничестве с Научно-исследовательским институтом Сан-Рафаэле, Милан, Италия.

Результаты: Почти все изоляты были идентифицированы как европейско-американские (96.9%); 18.5% изолятов оказались устойчивыми к фторхинолонам, но мутаций, придающих устойчивость к бедаквилину или линезолиду, выявить не удалось. Менее половины (43.3%) изолатов были сгруппированы (< 5 расстояния между SNPs) всего в семьи национальных кластерах, в то время как шесть изолатов были частью из разных трансграничных кластеров. Все кластерные случаи были из Болгарии.

Заключение: ПГС зарекомендовал себя как надёжный инструмент для эпиднадзора и отслеживания недавней передачи туберкулёза и обладает потенциалом для прогнозирования резистентности к большинству противотуберкулёзных препаратов.

Ключевые слова

множественная лекарственная устойчивость, передача