St. Petersburg Pasteur Institute, Russia
Laboratory of Molecular Epidemiology and Evolutionary Genetics

Activity report, 2021-2022: TUBERCULOSIS AND OTHER MYCOBACTERIA

Head of Laboratory: Igor Mokrousov

Permanent staff: Olga Narvskaya, Anna Vyazovaya, Daria Starkova, Alena Gerasimova, Daria Terentieva, Regina Mudarisova

PROJECTS AND COLLABORATIONS

Funded Projects

- Joint project with National Institute for Public Health and Environment (RIVM, Bilthoven, Netherlands) on drug resistant tuberculosis, 2018-2020 (co-PI: I. Mokrousov and R. Anthony). 2018-2021

– Russian Science Foundation, Project 19-14-00013 ("Uneven evolutionary and epidemic trajectory of the paradoxical ancient subtype of the East Asian lineage of Mycobacterium tuberculosis: stochastic fluctuations or causative correlations?" PI – Igor Mokrousov), 2019-2021. Funding continued for 2022-2023.

– Russian Science Foundation, Project 19-15-00028 ("Development of new efficient compounds against drug resistant Mycobacterium tuberculosis taking into account the population structure of the pathogen" PI – Anna Vyazovaya), 2019-2021.

- Project supported by PTR program of Institut Pasteur Paris "Transcriptional Response for AntimiCrobial Resistance detection in TB" (Coordinator - An van den Bossche, Belgium; Russian PI- Igor Mokrousov). 2019-2022.

- Russian Foundation for Basic Research, project 20-04-00686 "Deep machine learning methods in Mycobacterium tuberculosis genomics for the building of an open platform for the analysis of the pathogen's evolutionary signatures" (PI - E. Shitikov, Center of Physico-Chemical Medicine, Moscow), 2020-2022

- Russian Foundation for Basic Research, project 19-515-55009 (joint project co-funded by National Natural Science Foundation China) "Integral insight into development of drug resistant tuberculosis in adults versus children: impact of bacterial strain and surrounding microbiome" (PI - Dr Zhdanova, Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk, Russia), 2020-2022

- Russian Foundation for Basic Research project 20-515-80006 under BRICS STI Framework Programme Response to COVID-19 global pandemic (PI – Prof. Y. Schwartz, Novosibirsk Research Institute of Tuberculosis) 2021-2022

- Russian Science Foundation, project 22-15-00432 "The development of comprehensive mycobacterial diagnostics methods" (PI – Danila Zimenkov, Engelgardt's Institute of Molecular Biology RAS). 2022-2024.

International collaborations

National Institute for Public Health and the Environment, RIVM (2018-2021), Beijing Children's Hospital, China (2017-2021), Stephan Angeloff Institute of Microbiology and Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences (Sofia, Bulgaria), National TB Reference Laboratory, University Hospital Shefqet Ndroqi (Tirana, Albania), Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw (Poland), Instituto de Investigação do Medicamento, Faculdade de Farmácia, Universidade de Lisboa (Lisbon, Portugal).

National collaborations

Omsk State Medical University, Scientific Center of Family Health and Reproductive Problems (Irkutsk), Buryat State University (Ulan-Ude), Ural Research Institute of Phthisiopulmonology (Ekaterinburg), Northern Medical University (Archangelsk), Anti-tuberculosis dispensaries in Kaliningrad, Petrozavodsk (Karelia), Syktyvkar (Komi), Murmansk, Pskov, Omsk.

MAJOR RESEARCH RESULTS

Molecular monitoring of the Mycobacterium tuberculosis population in Murmansk region, Russia

In Murmansk oblast, the first molecular-genetic studies of circulating strains of *Mycobacterium tuberculosis* were carried out during the years of an increase in the incidence of tuberculosis (TB) (2003–2006). The study's aim was to carry out a genotypic characterization of the *M. tuberculosis* population in Murmansk oblast and analysis of changes in its structure over 15 years. Sixty-seven *M. tuberculosis* strains from patients with TB newly diagnosed in 2017 were studied. The strains were assigned to the Beijing genotype and its major clusters based on analysis of specific markers. The Beijing strains were typed by 24 MIRU–VNTR loci. All non-Beijing strains were subjected to spoligotyping. Genotypes of *M. tuberculosis* were identified: Beijing (52.2%), Ural (19.4%), T (9.0%), LAM (7.5%), Haarlem (3.0%), and X (1.5%). Among Beijing strains, the Central Asian/Russian cluster with heterogeneous MIRU–VNTR profiles was predominant—34.3% (23/67). Multiple drug resistance (MDR)—resistance to rifampicin and isoniazid caused by mutations *rpoB* Ser-531Leu (TCG → TTG) and *katG* Ser315Thr (AGC → ACC)—was detected in 26.9% of the strains; the largest proportion of MDR strains were found in the Beijing B0/W148 cluster (85.7%), represented mainly by the MIRU–VNTR profile 100-32. High levels of clustering of Beijing Central Asian/Russian (CR = 0.68) and B0/W148 (CR = 0.71) strains reflect their current dissemination. In 2003–2017, a steady dominance of the Beijing genotype with a tendency to an increase from 44.0 to 52.2% was observed. The proportion of MDR strains in the cluster B0/W148 Beijing increased by 3.5 times, which indicates the selection and accumulation of this epidemiologically and clinically unfavorable variant of the tuberculosis pathogen in Murmansk oblast.

Molecular Insight into Mycobacterium tuberculosis resistance to nitrofuranyl amides gained through metagenomics-like analysis of spontaneous mutants

(collaborative project with Institute of Organic Chemistry with Centre of Phytochemistry, and Stephan Angeloff Institute of Mcirobiology, Bulgarian Academy of Sciences)

The information on the nitrofuran mode of action on mycobacteria and the molecular mechanism of mycobacterial resistance to nitrofurans is limited. Different spontaneous mutations emerge in the *M. tuberculosis* population and may be selected and fixed if they are sufficiently beneficial for bacterial survival, adaptation, and fitness. In this study, we describe the synthesis of the new nitrofuranyl amides and investigate their anti-TB activity and possible mechanism of action/resistance through whole-genome sequencing of *M. tuberculosis* spontaneous mutants. We focused on nitrofuranyl amides since they possess strong antitubercular and antibacterial activity. However especially in case of antitubercular activity, their mechanism of action is still largely unknown.

Table 1. Genotypes and drug resistance of *M. tuberculosis* strains from Murmansk region in Russia

Genotype	Sensitive (n=41)	Mono/polyresistant (n=8)	MDR (n=18)	Total (n=67)
Beijing	17	3	15	35
Beijing B0/W148	0	1	6	7
Central-Asian/Russian	13	2	8	23
Beijing, other	4	0	1	5
Non-Beijing	24	5	3	32
Ural	9	4	0	13
T	6	0	0	6
LAM	5	0	0	5
Haarlem	1	1	0	2
X	1	0	0	1
Unknown	2	0	3	5

Methods. A series of six new nitrofuranyl amides was synthesized by reaction of 5-nitrofuran-2-carbonyl chloride with different amines. The *in vitro* activity was assessed on the reference strain *Mycobacterium tuberculosis* H37Rv. The most active compound **11** was further used for *in vitro* selection of the spontaneous resistant mutants. Strain H37Rv was cultured at the elevated concentrations of **11**, and DNA from pooled colonies was subjected to WGS followed by bioinformatics analysis.

Results. The same mutations in six genes were detected in bacterial cultures grown under increased concentrations of **11** (2x, 4x, 8x MIC). The mutant positions were presented as mixed wild type and mutant alleles while increasing the concentration of the compound led to a semi-proportional and significant (in 4 cases) increase of mutant alleles (see **Figure 1** as an example). The identified genes belong to different categories and pathways. Some of them were previously reported as mediating drug resistance or drug tolerance, and counteracting oxidative and nitrosative stress, in particular: *Rv0224c* (oxidative stress response), *fbiC* (F420 biosynthesis pathway that includes nitrofuran activating F420-dependent nitroreductase Ddn), *iniA* and lipase/esterase gene *Rv1592c* (efflux pump, maintenance of the plasma membrane integrity). Other genes were PhiRv1 phage protein *Rv1580c,* and conserved membrane protein *Rv1639c*. Five of six mutations were non-synonymous and some likely led to significant changes in protein structure. Gene-set interaction analysis revealed a certain weak interaction for gene pairs *Rv1592–Rv1639c* and *Rv1592–Rv0224c*.

Conclusions. In conclusion, this study experimentally demonstrated a complex multifaceted genetic response of *M. tuberculosis* to the action of the nitrofuranyl amide that concerned multiple genes and different pathways. Six genes contained mutations that emerged in bacterial cultures grown under increased concentrations of nitrofuran, furthermore, the increasing concentrations led to a higher proportion of the mutant alleles. The identified genes belong to different gene categories and pathways. The same mutations were detected in different independent experiments

that confirms a correlation between compound action and possible resistance mechanism. Furthermore, increasing the compound concentration of the compound led to a semi-proportional and significant (in 4 cases) increase of mutant alleles. Five of six mutations were non-synonymous and some could likely lead to significant changes in protein structure, especially mutations in *iniA* and *fbiC* with concordantly significant PAM1 and SIFT scores.

Further study should focus on experimental analysis of the role of the identified mutations, in particular by the transcriptomics and proteomics methods. Experiments focused on the interaction between nitrofurans and *M. tuberculosis* essential proteins in the presence and absence of a nitroreductase may shed light on the nitrofuran targets.

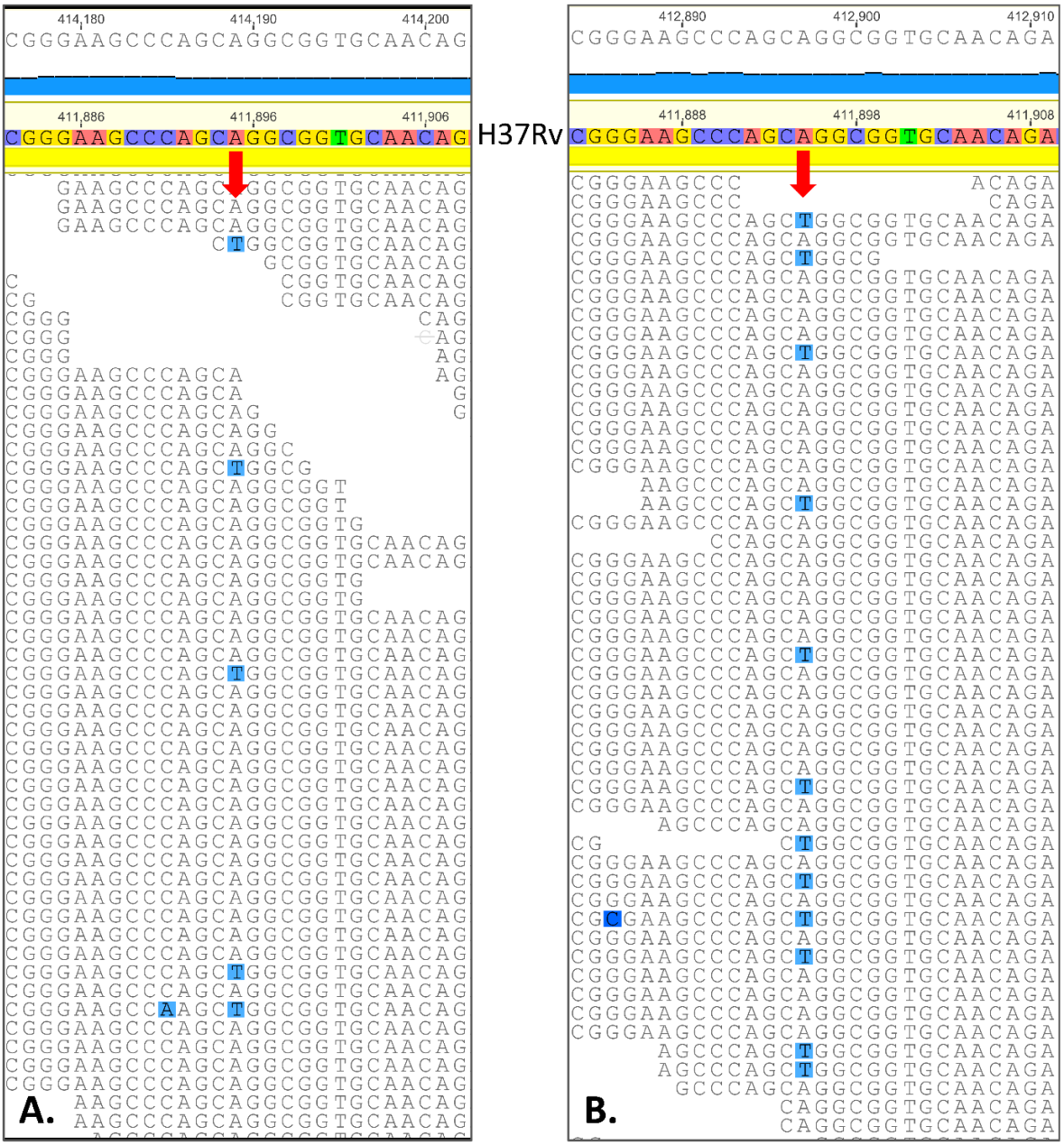

Figure 1. Example of mutant position (genome position 411895 A>T, *iniA* gene) with mixed wild type and mutant alleles, with increasing percent of mutant alleles in *M. tuberculosis* H37Rv subcultures grown under higher (2x and 8x MIC) concentrations of compound **11** – 1.0 μM (A) and 4.0 μM (B). Only part of the reads is shown that fit the Geneious window. The reads were aligned to the reference genome H37Rv (NC_000962.3). The red arrow indicates the position.

Extremely lethal and hypervirulent Mycobacterium tuberculosis strain cluster emerging in Far East, Russia

(collaborative project with St. Petersburg Research Institute of Phthisiopulmonology)

Previously, the C57BL/6 resistant mice infected with highly virulent *M. tuberculosis* strain were shown as a TB model reproducing an exacerbated inflammatory response in a resistant host to hypervirulent mycobacteria, leading to irreversible necrotic lung lesions. Properties of the Beijing strains were previously studied in this model, including ancient and modern sublineages from different countries. However, only modern Beijing sublineage strains from Russia were analyzed in those studies. In this study, we aimed to investigate the virulence properties of the *M. tuberculosis* strains of the recently described and MDR-associated ancient Beijing clusters from Russia in the C57BL/6 mouse model.

In the murine model, strains 396 (14717-15-cluster, from Buryatia, Far East) and 6691 (1071-32-cluster, from Omsk, Siberia) demonstrated contrasting properties. The 396-infected group had significantly higher mortality, more weight loss, higher bacterial burden, and more severe lung pathology. Furthermore, compared to the previously published data on other Russian epidemic Beijing strains (B0/W148, CAO, Central Asian Russian), strain 396 demonstrated the highest mortality.

Strain 6691 belongs to the Beijing 1071-32-cluster widespread across different FSU countries but at low prevalence and is relatively visible only in Omsk, Western Siberia (7%). This situation follows a traditional assumption that multiple drug resistance mutations reduce fitness and virulence. In contrast, highly lethal and hypervirulent strain 396 represents an intriguing Beijing 14717-15-cluster predominant only in Buryatia, Far East (16%), sporadically found beyond it, but not forming clusters of transmission. This specific case does not fit a theory of the highly virulent and highly transmitted strain. We term this cluster 14717-15 conditionally transmissible as it is endemically prevalent only in one location. The reasons may lie in the particular interplay of the human immune system and the genetic background of this strain, and further in-depth study is warranted.

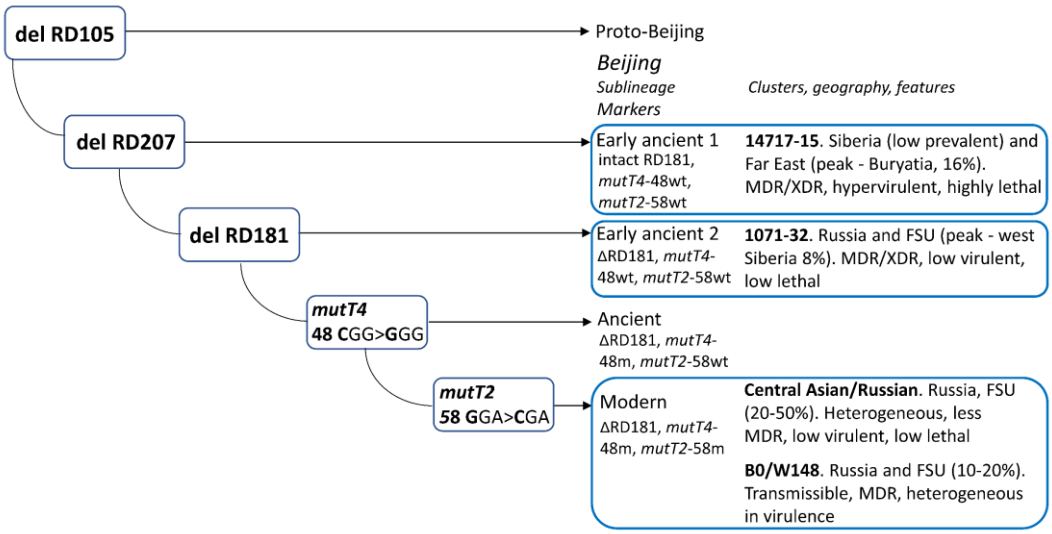

Figure 2. Simplified evolutionary pathway of the Beijing genotype with information on the above subtypes.

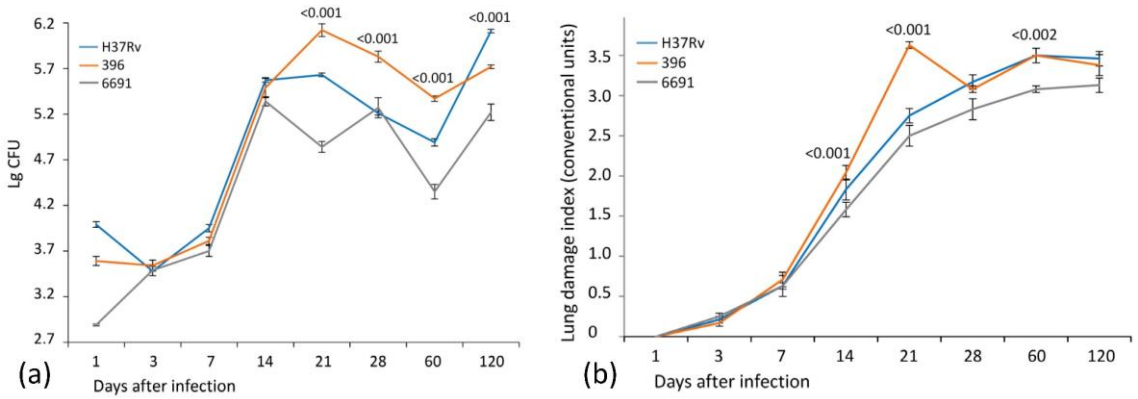

Figure 3. (a) Bacterial load in the lungs of mice infected with *M. tuberculosis* strains determined at different time points. (b). Lung pathology scores of mice infected with *M. tuberculosis* strains determined at different time points.

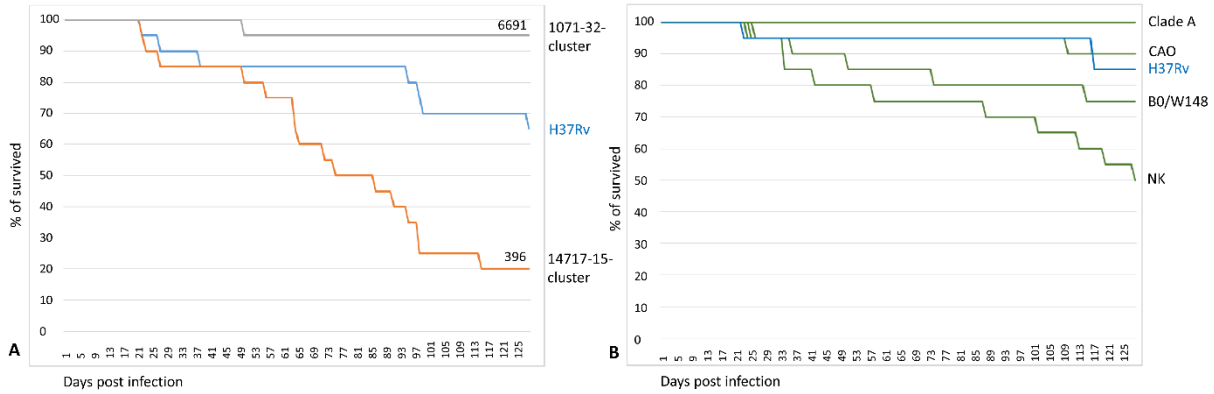

Figure 4. Comparison of survival of mice after infection with *M. tuberculosis* strains within 125 days p.i. in the similarly designed studies. The same strain H37Rv was used as reference. (a) ancient Beijing sublineage (this study). Strain 6691 belongs to 1071-32- cluster (RD181 deleted), strain 396 belongs to 14717-15-cluster (RD181 intact). (b) modern Beijing sublineage (adapted from Bespyatykh et al. [14]). B0/W148 strain is also named Russian epidemic or successful cluster. CladeA, CAO, NK strains belong to Central Asian Russian clade. The green color is used to show that all clinical isolates in this panel belong to the modern sublineage of the Beijing genotype.

Impact of pathobiological diversity of Mycobacterium tuberculosis on clinical features and lethal outcome of tuberculosis

(collaborative project with Omsk State Medical University and Clinical Tuberculosis Dispensary, Omsk)

Background. Recognition of the clinical significance of *Mycobacterium tuberculosis* population diversity is the key issue in molecular epidemiology and personalized medicine of tuberculosis (TB). Strains of different genetic lineages of *M. tuberculosis* demonstrate variability in some biological properties such as, in vitro growth rate, virulence in animal models, capacity to acquire drug resistance. The outcome of the disease, favorable or adverse, depends on a number of factors that act independently or synergistically and include not only the strain virulence, but also human genetics, HIV coinfection, immunosuppression, duration of tuberculosis disease, the timeliness of diagnosis, treatment efficacy, and social and environmental factors. *Mycobacterium tuberculosis* population in Russia is dominated by the notorious Beijing genotype which major variants are characterized by contrasting resistance and virulence properties. The simplified evolutionary pathway of the Beijing genotype with information on the above subtypes is shown in **Figure 2**.

Here we studied how these strain features could impact the progression of pulmonary tuberculosis (TB) concerning clinical manifestation and lethal outcome.

Results. Study collection included 548 *M. tuberculosis* isolates from 548 patients with newly diagnosed pulmonary TB in Omsk, West Siberia, Russia. Strains were subjected to drug susceptibility testing and genotyping to detect lineages, sublineages, and subtypes (within Beijing genotype). The Beijing genotype was detected in 370 (67.5%) of the studied strains. The strongest association with multidrug resistance (MDR) was found for epidemic cluster Beijing B0/W148 (modern sublineage) and two recently discovered MDR clusters 1071-32 and 14717-15 of the ancient Beijing sublineage. The group of patients infected with hypervirulent and highly lethal (in a mouse model) Beijing 14717-15 showed the highest rate of lethal outcome (58.3%) compared to Beijing B0/W148 (31.4%; P=0.06), Beijing Central Asian/Russian (29.7%, P=0.037), and non-Beijing (15.2%, P=0.001). The 14717-15 cluster mostly included isolates from patients with infiltrative but not with fibrous-cavernous and disseminated TB. In contrast, a group infected with low virulent 1071-32-cluster had the highest rate of fibrous-cavernous TB, possibly reflecting the capacity of these strains of prolonged survival and *chronicity* of the TB process.

Conclusions. This study indirectly shows that the traditional approach to assessing virulence and lethality in murine models does remain useful. The group infected with hypervirulent and highly lethal in murine model 14717-15 cluster had the highest rate of the lethal outcome (58.3%) compared to Beijing B0/W148 (31.4%), and non-Beijing (15.2%) groups.

In Russia, a country with a very high rate of primary MDR-TB, treatment is empirical and takes into account a high probability of primary MDR-TB for some genotypes. Now it is time to attempt considering other features of the infecting strains as well. Not only drug resistance but also strain virulence should be taken into consideration in personalized medicine and TB treatment.

Practical approach to detection and surveillance of emerging highly resistant Mycobacterium tuberculosis Beijing 1071-32-cluster

The recently discovered genetic variant *Mycobacterium tuberculosis* Beijing 1071-32 is characterized by multiple or extensive drug resistance. Beijing 1071-32 isolates were identified in Siberia (the most likely region of its origin), but also in European Russia, Central Asia, Transcaucasia, and unexpectedly, in the Balkan countries. We developed a molecular method for rapid detection of strains of this genotype and applied to the large collection of DNA.

Based on the phylogenetic analysis of the genomic data, authors identified three cluster-specific synonymous SNPs in the genes *Rv0144*, *Rv0373c*, and *Rv0334* and developed and validated the real-time PCR assay for their detection. Analysis of the genetically and geographically diverse collection of ~2400 M. tuberculosis isolates sampled in 1996 to 2020 (European and Asian parts of Russia, former Soviet Union countries, Albania, Greece, China, Vietnam, Japan and Brazil), confirmed 100% specificity and sensitivity of this assay.

All Beijing 1071-32 isolates carried a characteristic signature of six mutations that confer resistance to the four first-line antibiotics. Intriguingly, this combination includes the most frequent and efficient mutations (*rpoB450*, *katG315*, *rpsL43*), rare mutation (*embB497*), and compensatory mutations (*rpoC485*, *katG335*). The latter are supposed to restore the reduced strain fitness caused by previously acquired resistance mutations. The epistatic interaction of all these mutations could have influenced the spread of this genetic cluster.

In addition to the expected presence of the Beijing 1071-32 in Siberia and the European part of Russia, these strains were found in Central Asia, Transcaucasia, and also, quite unexpectedly, in the Balkan countries - Albania, Greece, Serbia. It is unknown whether the latter reflects already the local Balkan circulation of this strain

or independent events of its introduction from the countries of the former USSR (**Fig. 5**).

Enigmatically, all geographically distant isolates of this Beijing 1071-32 cluster have the same set of six resistance mutations. No intermediate strains with some, but not all these mutations were found to date. Speculatively, the acquisition of new mutations made the strain more adaptable compared to intermediate variants that subsequently disappeared from the population.

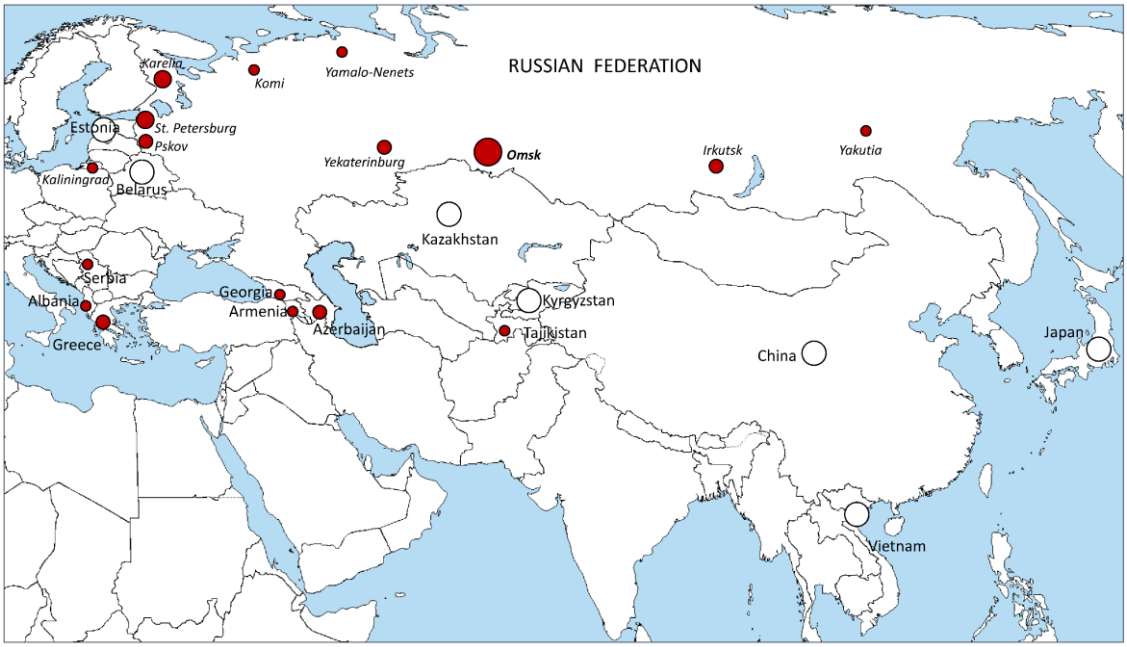

Figure 5. Geographic distribution of the Beijing 1071-32-cluster isolates identified in this study. Circle size roughly correlates with the proportion of identified isolates of this cluster (the smallest dots depict single isolates). Absence of these isolates in the local populations is shown by white circle.

Spatiotemporal dynamics of drug-resistant Mycobacterium tuberculosis in Estonia

(collaborative project with North Estonian Medical Centre Foundation and Estonian Tuberculosis Registry, Tallinn, Estonia)

Different and contrasting trends related to human migration and the implementation of health control programs influence the spread of drug-resistant tuberculosis (TB). We analyzed the *Mycobacterium tuberculosis* population structure in Estonia, a high-priority EU country for TB control, to detect the dynamic changes and underlying factors. The study collection included 278 *M. tuberculosis* isolates recovered in 1999 and 2014-2015. The isolates were subjected to drug susceptibility testing, genotyping, and analysis of sublineage/cluster-specific markers and drug resistance mutations. The Beijing genotype was the most prevalent and its rate increased from 28.6% in 1999 to 38.5% in 2015 (P=0.09). The non-Beijing strains represented Euro-American lineage (Latin American Mediterranean [LAM], Ural, Haarlem, T, X genotypes) and Indo-Oceanic lineage (one EAI-IND isolate) (**Fig. 6**). The proportion of isolates resistant to two or more drugs increased from 22.4% to 29.1% (P=0.1). The pre-XDR/XDR isolates were identified only within the Beijing genotype. In contrast, the drug resistance rate decreased in the LAM genotype from 42.1% to 11.8% (P=0.05). The Beijing B0/W148-cluster ("successful Russian strain") included only MDR, pre-XDR, or XDR isolates. All B0/W148-cluster isolates were resistant to two or more drugs compared to 28% of the Beijing 94-32-cluster (P=0.0002) (**Fig. 7**). The Beijing genotype was not identified in the isolates from patients born in

Estonia before 1940 compared to its 35.2% rate among other patients. In summary, the circulation of the highly drug-resistant isolates of the Beijing B0/W148 subtype, the increased prevalence of the Beijing genotype among HIV-coinfected patients, and the increased number of patients with alcohol abuse (47.5%) present major challenges of the current TB control in Estonia. The Beijing genotype was likely brought to Estonia after 1945 due to the massive human influx from the Soviet Union. In contrast, the main genotypes of the Euro-American lineage were likely endemic in Estonia during all 20th century.

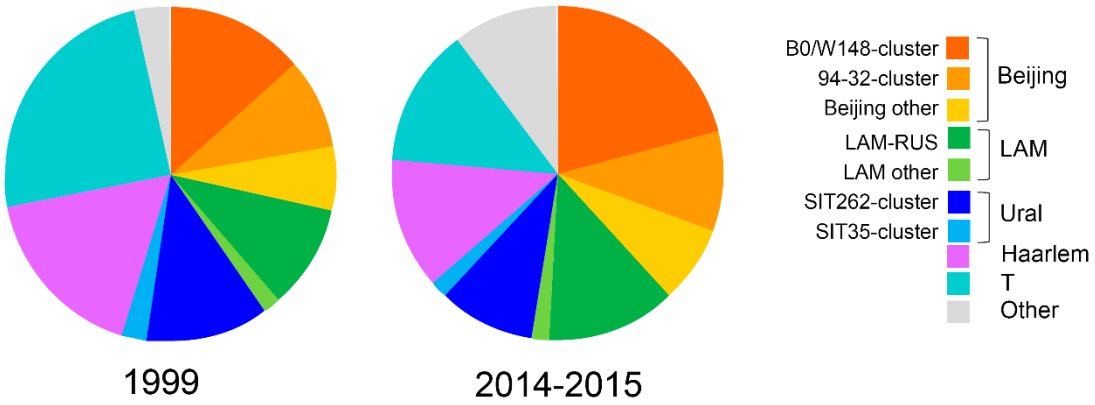

Figure 6. Prevalence of the main *M. tuberculosis* genotypes and subtypes in Estonia in 1999 and 2015.

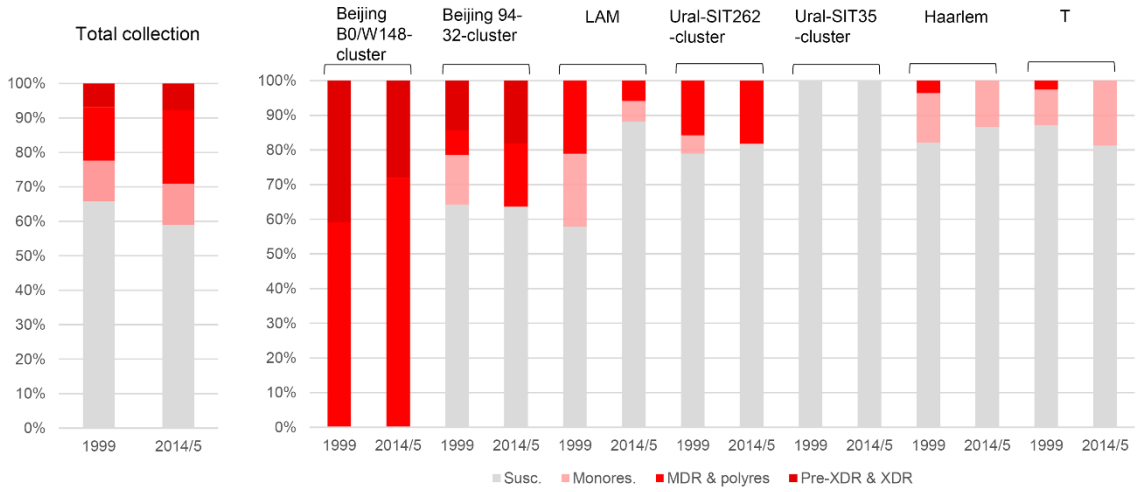

Figure 7. Drug resistance in the main *M. tuberculosis* genotypes in Estonia in 1999 and 2015.

Drug resistance of non-tuberculous mycobacteria in Northwestern Russia

Among a large group of nontuberculous mycobacteria (NTM) (more than 150 species), slow-growing bacteria of the MAC complex (*Mycobacterium avium* complex) - *M. avium* and *M. intracellulare* have been recognized as clinically most important pathogens. Being the causative agents of pulmonary mycobacteriosis, MAC can cause lung destruction in immunocompetent individuals and a disseminated form of infection in HIV-infected people. The aim of the study was to study the drug resistance of *M. avium* and *M. intracellulare* isolates isolated from patients with mycobacteriosis in the Northwestern Russia. is the most frequent etiology of disease

Materials and methods. For the period from 2014 to 2020, 192 slow-growing MAC isolates (164 - *M. avium*, 28 - *M. intracellulare*) from HIV-negative patients with

pulmonary disease were studied. Of the 164 *M. avium* isolates, 116 were isolated from newly diagnosed patients, 48 from previously treated patients (with an unknown treatment regimen). All *M. intracellulare* isolates were obtained from newly diagnosed patients. Drug susceptibility testing was performed using Sensititre SLOMYCO panels (Thermo Fisher Scientific). For clarithromycin, amikacin, moxifloxacin, and linezolid, the Clinical and Laboratory Standards Institute (CLSI) breakpoints have been used to interpret MIC values (CLA : S ≤ 8 mcg/ml, I = 16 mcg/ml, R ≥ 32 mcg/ml ; MXF : S ≤ 1 mcg/ml, I = 2 mcg/ml, R ≥ 4, mcg/ml ; LZD : S ≤ 8 mcg/ml, I = 16 mcg/ml, R ≥ 32 mcg/ml ; AMI : S ≤ 16 mcg/ml, I = 32 mcg/ml, R ≥ 64 mcg/ml).

Results. Of the four antibiotics (clarithromycin, moxifloxacin, linezolid, amikacin), clarithromycin was the most effective against both *M. avium* (67.1%; 110/164) and *M. intracellulare* (60.7%; 17/28) without significant difference in susceptible rate between the species ($p>0.05$). Overall, 57.3% *M. avium* and 53.5% *M. intracellulare* isolates were susceptible to linezolid. For moxifloxacin, 26.8% *M. avium* and 14.3% *M. intracellulare* isolates were susceptible; for amikacin - 57.3% *M. avium* and 53.5% *M. intracellulare* isolates were susceptible, respectively. Resistance rates to all antibiotics was higher in *M. intracellulare* compared to *M. avium*, but non-significantly ($p>0.05$). The rate of CLA-susceptible *M. avium* isolates was 20% higher in newly diagnosed patients compared to previously treated patients ($\chi2=6.296$; $p=0.013$). The rates of *M. avium* first isolates group susceptible to moxifloxacin, linezolid, and amikacin was also higher than in group of follow-up isolates.

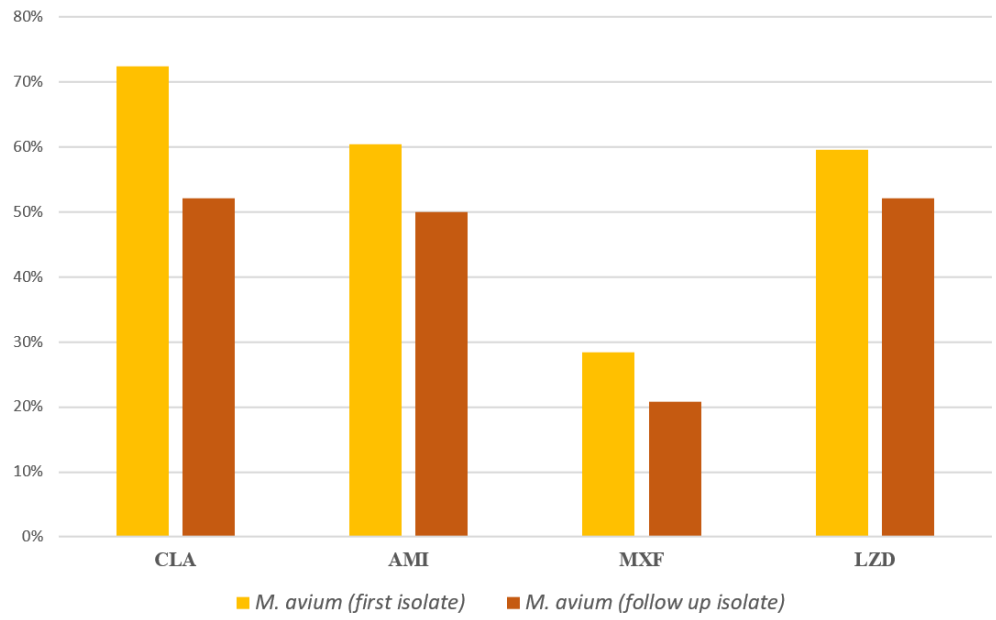

Figure 8. The distribution of proportions of *M. avium* strains sensitive to CLA (clarithromycin), AMI (amikacin), MXF (moxifloxacin) and LZD (linezolid) isolated from newly diagnosed (n=116) and previously treated patients (n=48).

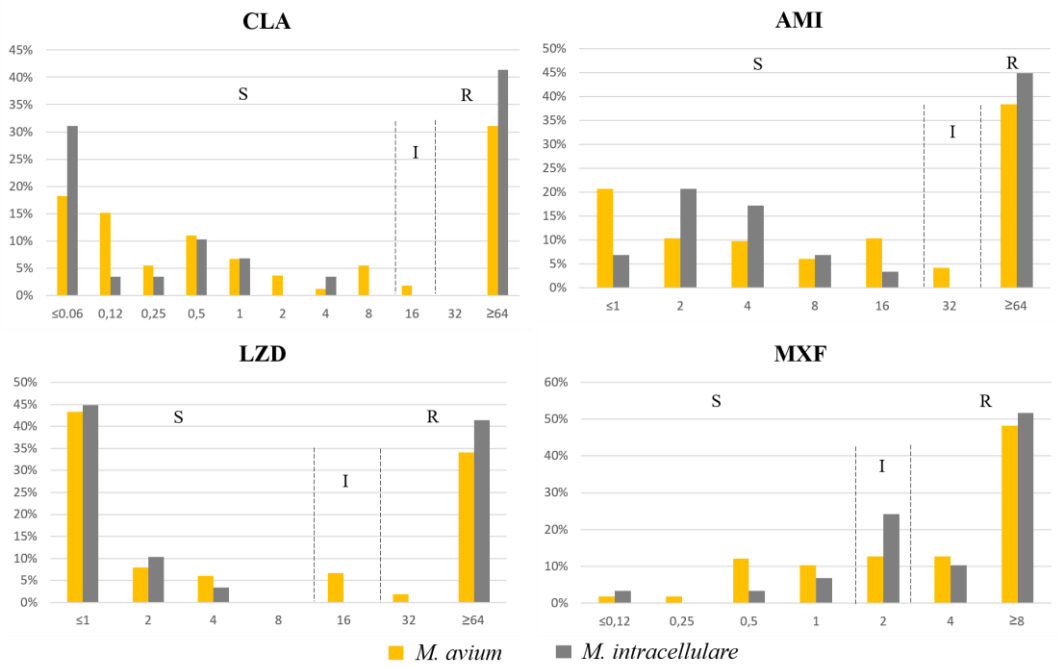

Figure 9. MIC distribution for CLA (clarithromycin), AMI (amikacin), MXF (moxifloxacin) and LZD (linezolid) in *M. avium* (n=164) and *M. intracellulare* (n=28) clinical isolates. The vertical dotted lines represent the MIC breakpoints for susceptible (S), intermediate (I), and resistant (R) MAC strains.

PUBLICATIONS

BOOK CHAPTER

Mokrousov I. Major Impact of Massive Migration on Spread of Mycobacterium tuberculosis Strains. In: Human Migration (Eds M de Lourdes Moreno and M.H. Crawford). Oxford University Press. 2021. pp. 255-267. ISBN: 9780190945961. DOI: 10.1093/oso/9780190945961.003.0020

Articles in peer-reviewed journals (Pubmed, Web of Science)

ARTICLES IN ENGLISH: 15 (9 - in Q1 [Web of Science])

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2. Vyazovaya A, Felker I, Schwartz Y, Mokrousov I. Population structure of Mycobacterium tuberculosis from referral clinics in Western Siberia, Russia: Before and during the Covid-19 pandemic // Infect Genet Evol. 2022 Sep;103:105343. doi: 10.1016/j.meegid.2022.105343.

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