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

Activity report, 2023-2024: TUBERCULOSIS AND OTHER MYCOBACTERIA

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PROJECTS AND COLLABORATIONS

Funded Projects

– 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, 2019-2023.

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

– Russian Science Foundation, project 24-44-00004 "Multifaceted adaptation of *Mycobacterium tuberculosis* to new-generation antibiotics at population and individual levels and impact on transmission", PI – Igor Mokrousov, 2024-2026. This is joint project with Children's Hospital Affiliated to Zhengzhou University, China (Chinese PI – Adong Shen), supported by National Natural Science Foundation of China.

International collaborations

Baoding Hospital/Beijing Children's Hospital, Zhengzhou Children's Hospital, China. Stephan Angeloff Institute of Microbiology, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria. National Reference Laboratory of Tuberculosis, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria. Laboratório de Biologia Molecular Aplicada a Micobactérias, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil. Almaty Branch of National Center for Biotechnology in Central Reference Laboratory, Almaty, Kazakhstan. Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de la Guadeloupe, Guadeloupe, France. Department of Medical Microbiology, Institute of Microbiology, University of Warsaw, Poland. Instituto de Investigação do Medicamento, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal.

National collaborations

St. Petersburg Research Institute of Phthisiopulmonology. Omsk State Medical University. Scientific Center of Family Health and Reproductive Problems (Irkutsk). Buryat State University (Ulan-Ude). Northern Medical University (Archangelsk). Anti-tuberculosis dispensaries in Kaliningrad, Petrozavodsk (Karelia), Syktyvkar (Komi), Murmansk, Pskov, Novgorod, Omsk, Ulan-Ude. Resource Center "Bio-bank Center", Research Park of St. Petersburg State University,

St. Petersburg. Department of Biomedicine and Genomics, Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency, Moscow. Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow. Mikloukho-Maklay Institute of Ethnology and Anthropology, Russian Academy of Sciences, Moscow. Scientific Research Center "Baikal Region", Irkutsk State University, Irkutsk.

MAJOR RESEARCH RESULTS

Molecular characteristics of fluoroquinolone-resistant *Mycobacterium tuberculosis* strains from newly diagnosed tuberculosis patients in the North-West of Russia

Fluoroquinolones remain the key second-line anti-tuberculosis drugs. The purpose of the study was the molecular characterization of fluoroquinolone-resistant *Mycobacterium tuberculosis* strains from newly diagnosed tuberculosis patients in the North-West of the Russian Federation.

The retrospective study collection included *M. tuberculosis* isolates isolated in 2015-2019 from previously untreated tuberculosis patients. Susceptibility to antituberculosis drugs (including the fluoroquinolone ofloxacin) was determined using the BACTEC MGIT960 or absolute concentration method. Mutations in the *gyrA* gene as a marker of resistance to fluoroquinolones, were detected using real-time PCR. Beijing genotype and its subtypes were detected by PCR and real-time PCR methods. Non-Beijing strains were spoligotyped.

Phenotypic resistance to ofloxacin was detected in 6.7% (40/599) of strains and in 17.4% (40/230) of MDR strains. 34 of 40 (85%) ofloxacin-resistant strains belonged to the Beijing genotype. 18 (45%) strains were assigned to the Russian epidemic subtype Beijing B0/W148 and 12 (30%) to Beijing Central Asian/Russian. The remaining 6 ofloxacin-resistant strains belonged to the Euro-American phylogenetic lineage. Mutations in the *gyrA* gene were found in 97.5% (39/40) of strains. The most common were mutations in codon 94 (69.2%, 27/39). The Asp94Gly substitution was in 57.5% (23/40) of ofloxacin-resistant strains and was dominant among Beijing (19/34) and non-Beijing (4/6) strains. The second most common substitution was Ala90Val (25%, 10/40). More than half of the ofloxacin-resistant strains Beijing B0/W148 (10/18) and Central Asian/Russian (7/12) carried the Asp94Gly mutation.

Conclusion. In the North-West of Russia in 2016-2019, primary resistance of *M. tuberculosis* to fluoroquinolones was 6.7% in the total collection and 17.4% of MDR strains, and was mainly caused by the *gyrA* Asp94Gly and Ala90Val mutations. Beijing B0/W148 genotype was characterized by the largest proportion of fluoroquinolone-resistant strains.

Table 1. Spoligoprofiles of 40 ofloxacin-resistant *M. tuberculosis* strains

SIT, family	43-spoligoprofile	Number of strains	*gyrA* mutation
SIT1 Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	34	19 - Asp94Gly 8 - Ala90Val 3 - Asp94Ala 2 - Ser91Pro 1 - нет
SIT42 LAM	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	2	1 - Ala90Val 1 - Asp94Gly
SIT252 LAM	■■■■■■■■■■□□■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	1	Asp94Gly
SIT4 LAM	□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■□□□□■■■■■■■	1	Asp94Gly
SIT53 L4-unclassified	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	Asp94Gly
SIT251 L4-unclassified	■■■■■■■□□□□□□□□□□□□□□□□□□□□□□■■■■■□□□□■■■■■■■	1	Ala90Val

Genetic Diversity and Primary Drug Resistance of *Mycobacterium tuberculosis* Beijing Genotype Strains in Northwestern Russia

The Beijing genotype is the main family of *Mycobacterium tuberculosis* in Russia. We analyzed its diversity and drug resistance in provinces across Northwestern Russia to identify the epidemiologically relevant Beijing strains. The study collection included 497 isolates from newly-diagnosed tuberculosis (TB) patients. Bacterial isolates were subjected to drug-susceptibility testing and genotyping. The Beijing genotype was detected in 57.5% (286/497); 50% of the Beijing strains were multidrug-resistant (MDR). Central Asian/Russian and B0/W148 groups included 176 and 77 isolates, respectively. MDR was more frequent among B0/W148 strains compared to Central Asian/Russian strains (85.7% vs. 40.3%, $p < 0.0001$). Typing of 24 minisatellite loci of Beijing strains revealed 82 profiles; 230 isolates were in 23 clusters. The largest Central Asian/Russian types were 94-32 ($n = 75$), 1065-32 ($n = 17$), and 95-32 ($n = 12$). B0/W148 types were 100-32 ($n = 59$) and 4737-32 ($n = 5$) (Figure 1). MDR was more frequent in types 1065-32 (88.2%), 100-32 (83.1%), and 4737-32 (100%). In contrast, type 9391-32 ($n = 9$) included only drug-susceptible strains. To conclude, *M. tuberculosis* Beijing genotype is dominant in Northwestern Russia, and an active transmission of overwhelmingly MDR B0/W148 types explains the reported increase of MDR-TB. The presence of MDR-associated minor variants (type 1071-32/ancient Beijing and Central Asia Outbreak strain) in some of the studied provinces also requires attention.

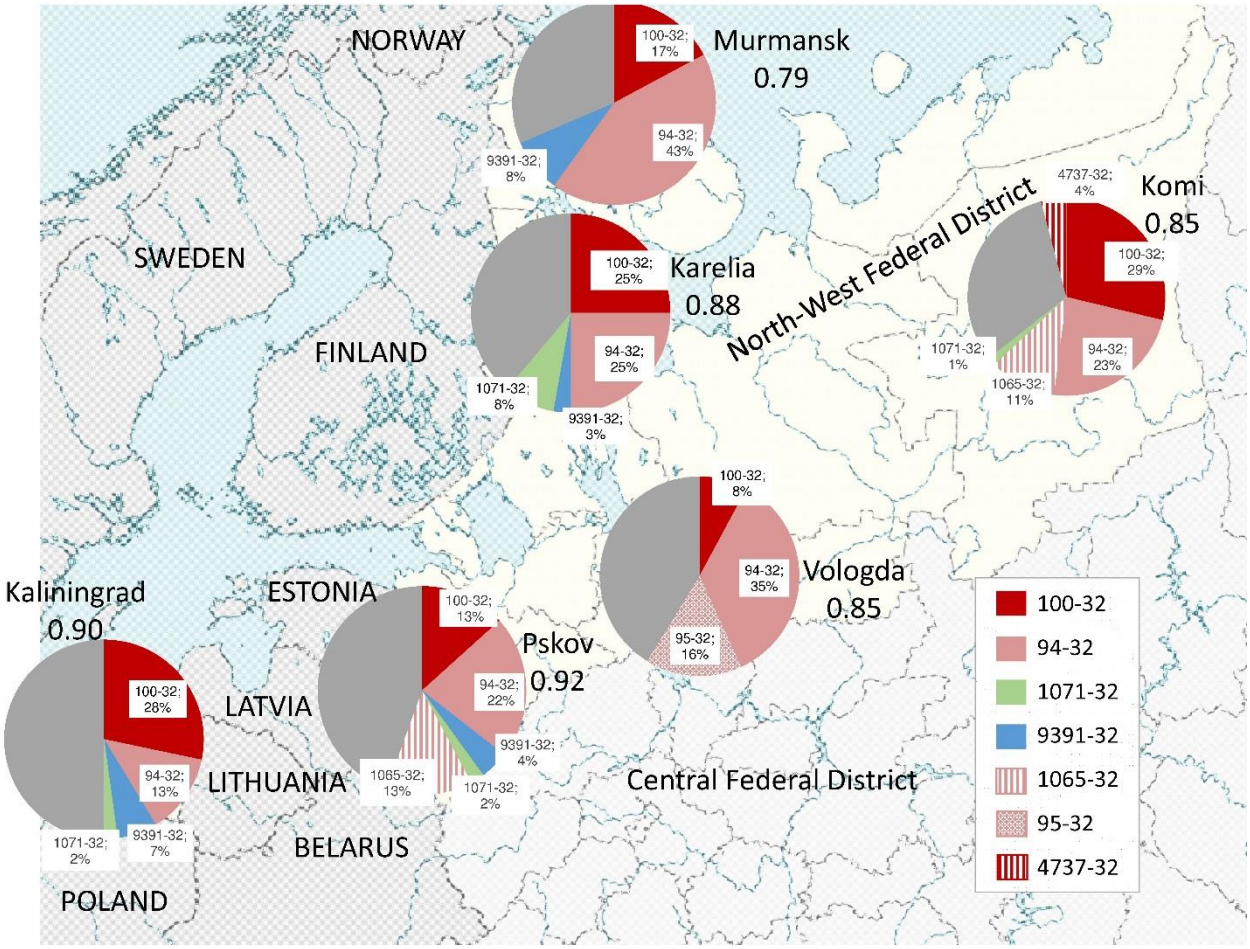

Figure 1. Distribution of the Beijing genotype VNTR types in northwestern Russia.

Increasing circulation of multi-drug resistant tuberculosis strains in Buryatia, high-burden and ethnically diverse region in the Russian Far East

Buryatia is a multidrug-resistant tuberculosis (MDR-TB) high-burden region in the Russian Far East with ethnically diverse population (30 % Mongoloid Buryats and 65 % Russians). Two hundred M. tuberculosis strains from newly-diagnosed patients were subjected to phenotypic testing and genotyping. The Beijing genotype was more prevalent among Russians than Buryats (68 % vs 53 %; P = 0.055). European non-Beijing genotypes (LAM, Ural, Haarlem) were double more prevalent in Buryats vs Russians (39.2 % vs 20.5 %; P = 0.01). Higher prevalence of Beijing among former prison inmates (79 % vs 61 % in other patients, P = 0.1) suggests its increased transmissibility. The Russian epidemic cluster B0/W148 was in 9.5 %, double smaller than elsewhere in Siberia. The hypervirulent Beijing 14717-15-cluster was endemic in Buryatia but paradoxically enough, it was more frequently isolated from Russians than Buryats (9.1 % vs 3.9 %; P = 0.2). Beijing subtypes B0/W148, CAO, and 14717-15 were associated with poly/multi-drug resistance (P = 0.01-0.0001) (Fig. 2). HIV coinfection was more frequent in Russians than in Buryats: 35/141 (24.8 %) vs 5/51 (9.8 %), P = 0.03. To conclude, M. tuberculosis population structure in Buryatia retained its singularities compared to other parts of Russia and remains strikingly different from the neighboring Mongolia (Fig. 3). A circulation of strongly MDR-associated Beijing subtypes and drug-resistant non-Beijing strains highlights a risk of their broader dissemination.

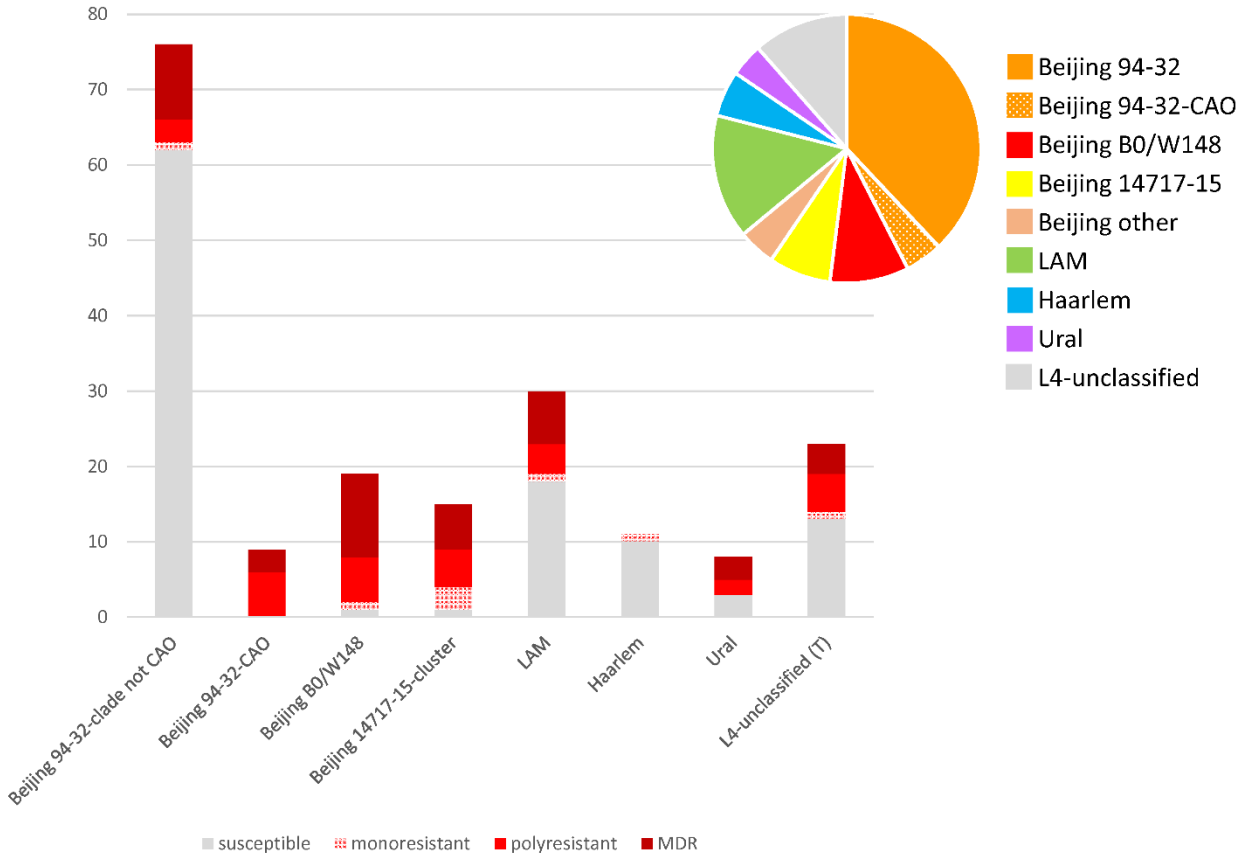

Figure 2. Drug resistance in *M. tuberculosis* genotypes/subtypes in Buryatia.

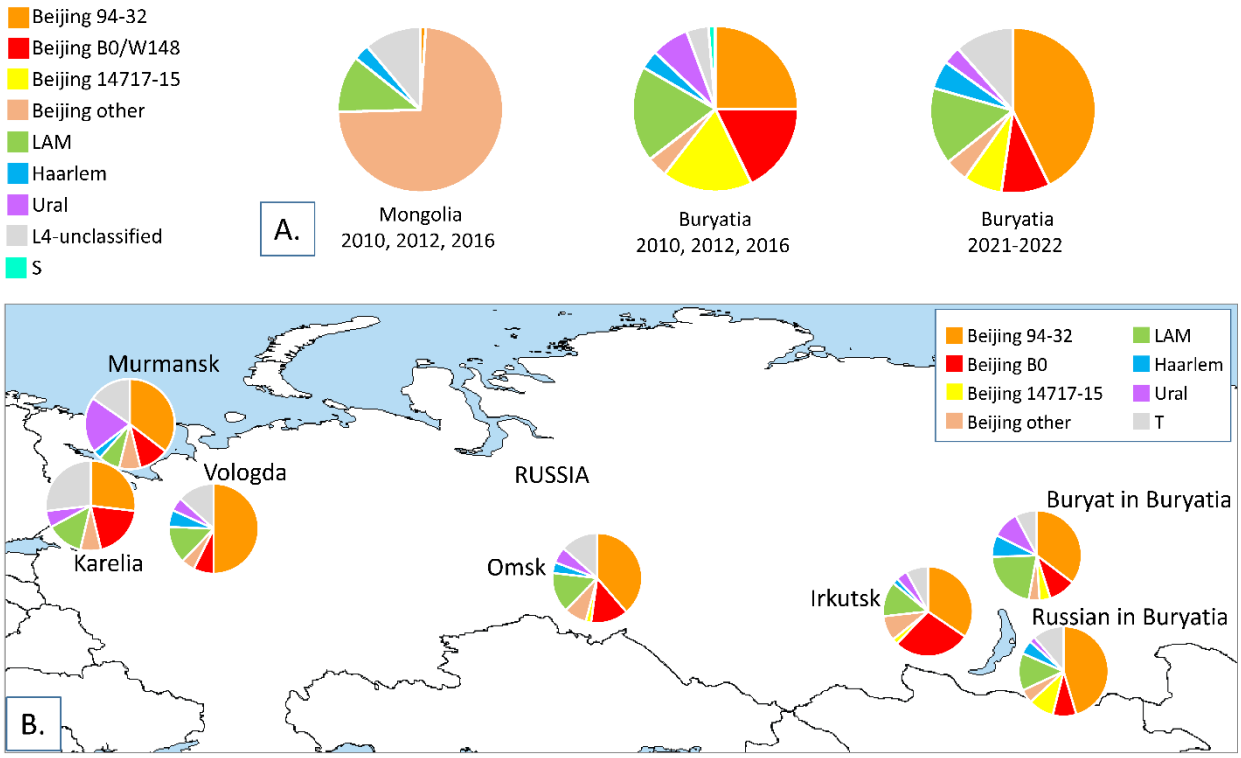

Figure 3. *M. tuberculosis* genotypes/subtypes in Buryatia and other Russian regions.

Insight into pathogenomics and phylogeography of hypervirulent and highly-lethal Mycobacterium tuberculosis strain cluster

The Mycobacterium tuberculosis Beijing genotype is globally spread lineage with important medical properties that however vary among its subtypes. M. tuberculosis Beijing 14717-15-cluster was recently discovered as both multidrug-resistant, hypervirulent, and highly-lethal strain circulating in the Far Eastern region of Russia. Here, we aimed to analyze its pathogenomic features and phylogeographic pattern.

The study collection included M. tuberculosis DNA collected between 1996 and 2020 in different world regions. The bacterial DNA was subjected to genotyping and whole genome sequencing followed by bioinformatics and phylogenetic analysis. The PCR-based assay to detect specific SNPs of the Beijing 14717-15-cluster was developed and used for its screening in the global collections. Phylogenomic and phylogeographic analysis confirmed endemic prevalence of the Beijing 14717-15-cluster in the Asian part of Russia, and distant common ancestor with isolates from Korea (> 115 SNPs) (Fig. 4). The Beijing 14717-15-cluster isolates had two common resistance mutations RpsL Lys88Arg and KatG Ser315Thr and belonged to spoligotype SIT269. The Russian isolates of this cluster were from the Asian Russia while 4 isolates were from the Netherlands and Spain. The cluster-specific SNPs that significantly affect the protein function were identified in silico in genes within different categories (lipid metabolism, regulatory proteins, intermediary metabolism and respiration, PE/PPE, cell wall and cell processes).

To conclude, we developed a simple method based on real-time PCR to detect clinically significant MDR and hypervirulent Beijing 14717-15-cluster. Most of the identified cluster-specific mutations were previously unreported and could potentially be associated with increased pathogenic properties of this hypervirulent M. tuberculosis strain. Further experimental study to assess the pathobiological role of these mutations is warranted.

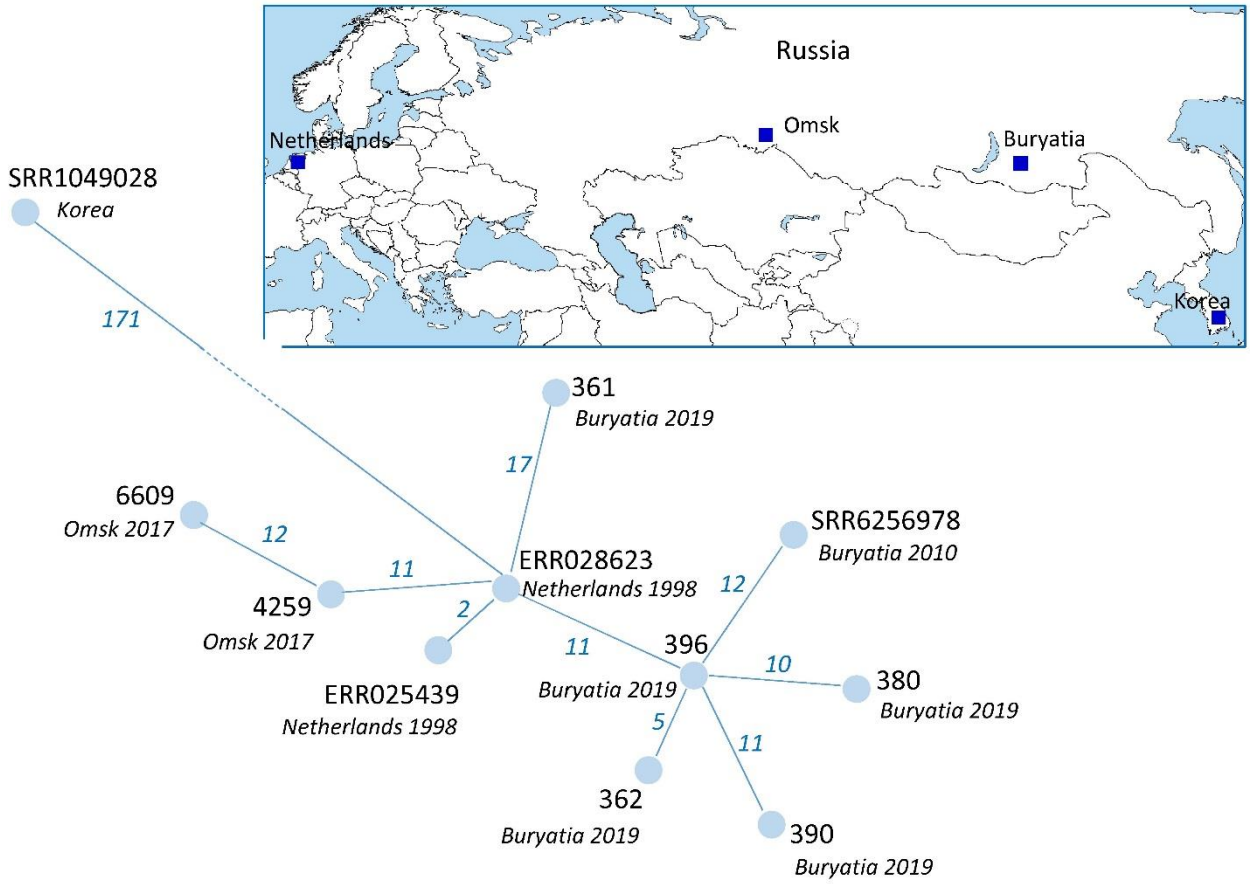

Figure 4. Phylogenomic analysis of the Beijing 14717-15-cluster strains. Strain from Korea is an outgroup.

A multifaceted interplay between virulence, drug resistance, and the phylogeographic landscape of *Mycobacterium tuberculosis*

Latin-American Mediterranean (LAM) family is one of the most significant and global genotypes of *Mycobacterium tuberculosis*. Here, we used the murine model to study the virulence and lethality of the genetically and epidemiologically distinct LAM strains. The pathobiological characteristics of the four LAM strains (three drug resistant and one drug susceptible) and the susceptible reference strain H37Rv were studied in the C57BL/6 mouse model. The whole-genome sequencing was performed using the HiSeq Illumina platform, followed by bioinformatics and phylogenetic analysis. The susceptible strain H37Rv showed the highest virulence. Drug-susceptible LAM strain (spoligotype SIT264) was more virulent than three multidrug-resistant (MDR) strains (SIT252, SIT254, and SIT266). All three MDR isolates were low lethal, while the susceptible isolate and H37Rv were moderately/highly lethal. Putting the genomic, phenotypic, and virulence features of the LAM strains/spoligotypes in the context of their dynamic phylogeography over 20 years reveals three types of relationships between virulence, resistance, and transmission (Fig. 5). First, the most virulent and more lethal drug-susceptible SIT264 increased its circulation in parts of Russia. Second, moderately virulent and pre-XDR SIT266 was prevalent in Belarus and continues to be visible in North-West Russia. Third, the low virulent and MDR strain SIT252 previously considered as emerging has disappeared from the population. These findings suggest that strain virulence impacts the transmission, irrespective of drug resistance properties. The increasing circulation of susceptible

but more virulent and lethal strains implies that personalized TB treatment should consider not only resistance but also the virulence of the infecting *M. tuberculosis* strains.

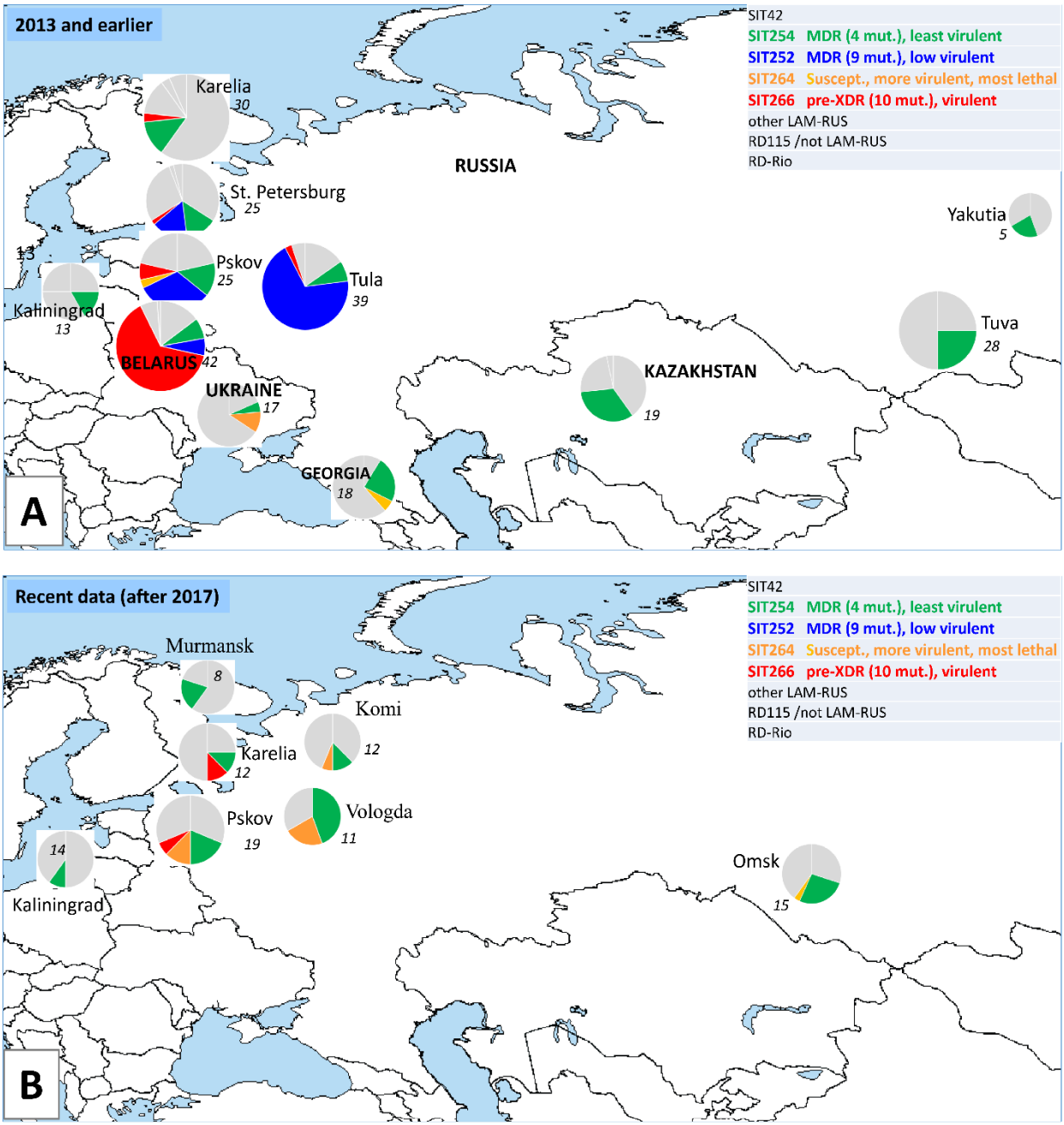

Figure 5. Dynamic changes in distribution of the studied LAM spoligotypes in Russia and neighbors.

Mouse model of experimental tuberculosis and chemotherapy

In spite of well-known limitations, mice remain useful as model animals to study tuberculosis (TB) pathogenesis. Here, we used the C57BL/6 mouse model and intravenous tail injection of the bacterial suspension according to the well-established and validated methodology previously used by us and others to study *M. tuberculosis* virulence. The M. tuberculosis strains represented the most epidemically relevant global genotypes, Beijing and LAM, and were previously comprehensively characterized by drug susceptibility testing, whole genome sequencing (WGS)

and in the same murine model of intravenous tail injection. All strains were MDR or pre-XDR but differed in virulence and lethality in mice. C57BL/6 mice were infected with different strains. Two animal groups per each strain (each group included 20 mice) were observed during 200 days. The first infected group was a control one, without treatment. The second infected group received an adequate chemotherapy regimen based on the knowledge of the phenotypic and genotypic resistance of the infecting strains. The anti-tuberculosis therapy regimen consisted of 4 new-generation or repurposed drugs: moxifloxacin (7 mg/kg), linezolid (10 mg/kg), bedaquiline (6.7 mg/kg - 3.9 mg/kg) and perchlozone (12 mg/kg). All drugs were administered orally 5 times a week during 177 days.

After 77 and 177 days of anti-tuberculosis therapy, animals (6 per each strain-determined group of mice) were euthanized and examined for different pathology-related characteristics: lung weight coefficient, lung lesions, bacterial load of the lungs, Histology. Treatment efficiency index was calculated. WGS of the isolates recovered from mice after 2 and 5.5 months of treatment. From each strain-determined group there were 5 to 6 mice/isolates analyzed at each time point. WGS data were analysed using SAM-TB and Geneious to detect possible resistance mutations in M. tuberculosis, linked to resistance to the administered drugs in genes *gyrA, gyrB* (fluoroquinolones resistance), *rplC, rrs, rrl* (linezolid resistance), *Rv0678, atpE, pepQ*, and *Rv1979c* (bedaquiline resistance).

As a result, a full course of correct and continuous treatment (5.5 months) significantly reduced the contamination of the lungs with Mycobacterium tuberculosis. A significant effect of anti-tuberculosis therapy was recorded in terms of bacterial burden, lung lesions and mice survival.

Survival analysis shows that no mice died in three of the four strain-determined groups that received chemotherapy (Fig. 6). Only in one group of mice infected with hypervirulent and highly-lethal strain 396, the lethal cases were noted but significantly less frequently compared to the untreated group.

Mutations associated with resistance to the administered drugs were not detected even in a small percentage in strains isolated from the lungs of mice after 2 and 5.5 months of treatment. This finding is remarkable given a known emergence of resistance mutations in consecutive isolates recovered from human patients during long-term treatment, e.g. with bedaquiline. However, in real life, unfortunately, human patients may and do have multiple episodes of interrupted chemotherapy for any reason, and this makes a great difference compared to the fully compliant model animals receiving scientifically adequate chemotherapy.

We conclude that correct treatment and complete compliance resulted in efficient treatment and overall positive outcome in terms of drastically reduced lethality in all treated groups of animals. This finding highlights that early diagnosis including detection of resistance determinants, hence early choice of correct chemotherapy regimen and patient compliance are the key factors to stop TB.

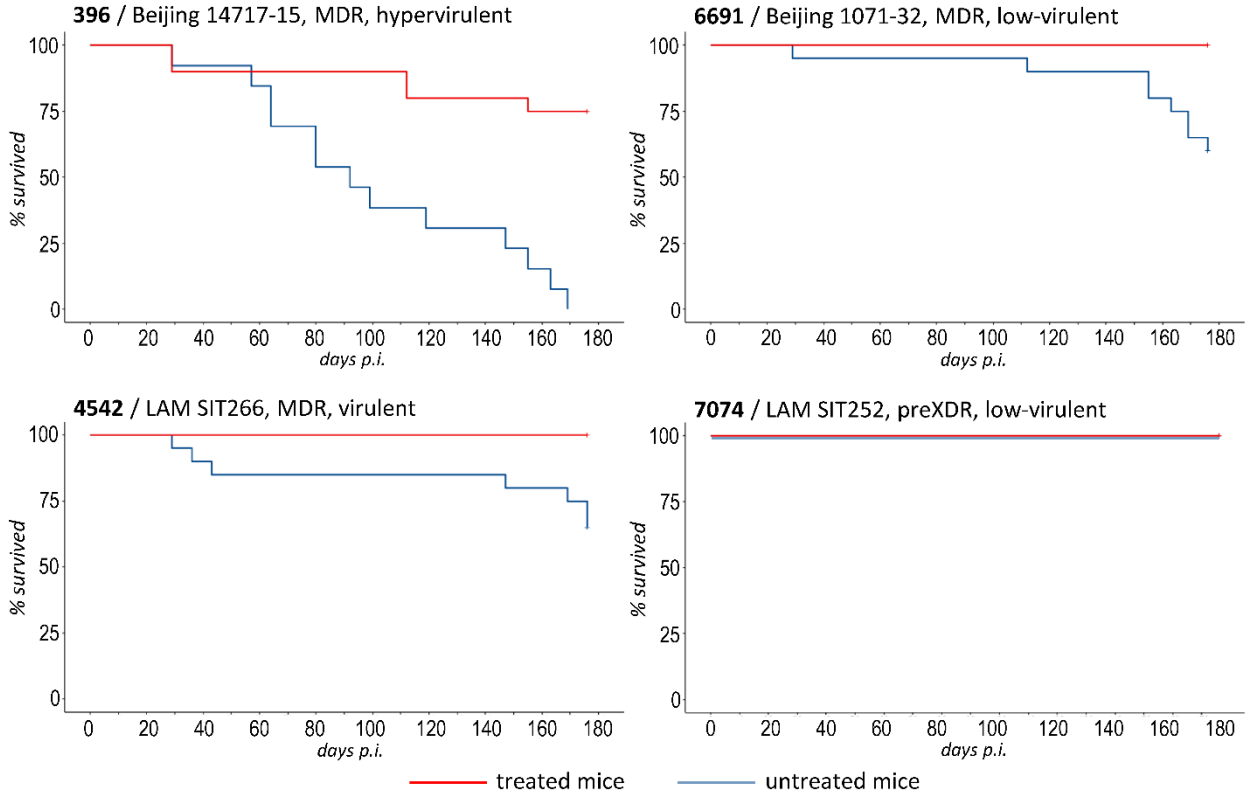

Figure 6. Survival of mice infected with different Beijing and LAM strains who were not treated or received correct chemotherapy.

Origin and dispersal of the Mycobacterium tuberculosis Haarlem genotype: Clues from its phylogeographic landscape and human migration

The Haarlem family belongs to the Euro-American phylogenetic lineage of *Mycobacterium tuberculosis* and is one of the globally spread genotypes of this important human pathogen. In spite of the sporadic observations on drug resistance and peculiar virulence profile, Haarlem remains in the shade of other *M. tuberculosis* genotypes. We analyzed genotyping data of the Haarlem genotype in light of its pathogenic properties and relevant human migration, to gain insight into its origin, evolutionary history, and current spread. Central Europe is marked with a very high prevalence of both major Haarlem subclades ancestral H3/SIT50 and derived H1, jointly making 33-41% in Czechia, Austria, and Hungary. There is a declining gradient of Haarlem beyond central Europe with 10-18% in Italy, France, Belgium, 10-13% in the Balkan countries and Turkey. Placing the available genetic diversity and ancient DNA data within the historical context, we hypothesize that *M. tuberculosis* Haarlem genotype likely originated in Central Europe and its primary long-term circulation occurred within the area of the former Austria/Austria-Hungary Empire in the 14th-19th centuries (Fig. 7). The genotype is not highly transmissible and its spread was driven by long-term human migration. The European colonial expansion (when accompanied by a sufficient volume of migration) was a vehicle of its secondary dissemination. We conclude that human migration and its lack thereof (but not strain pathobiology) was a major driving force that shaped the population structure of this global lineage of *M. tuberculosis*. At the same time, Haarlem strains appear over-represented in some ethnic groups which warrants in-depth experimental research.

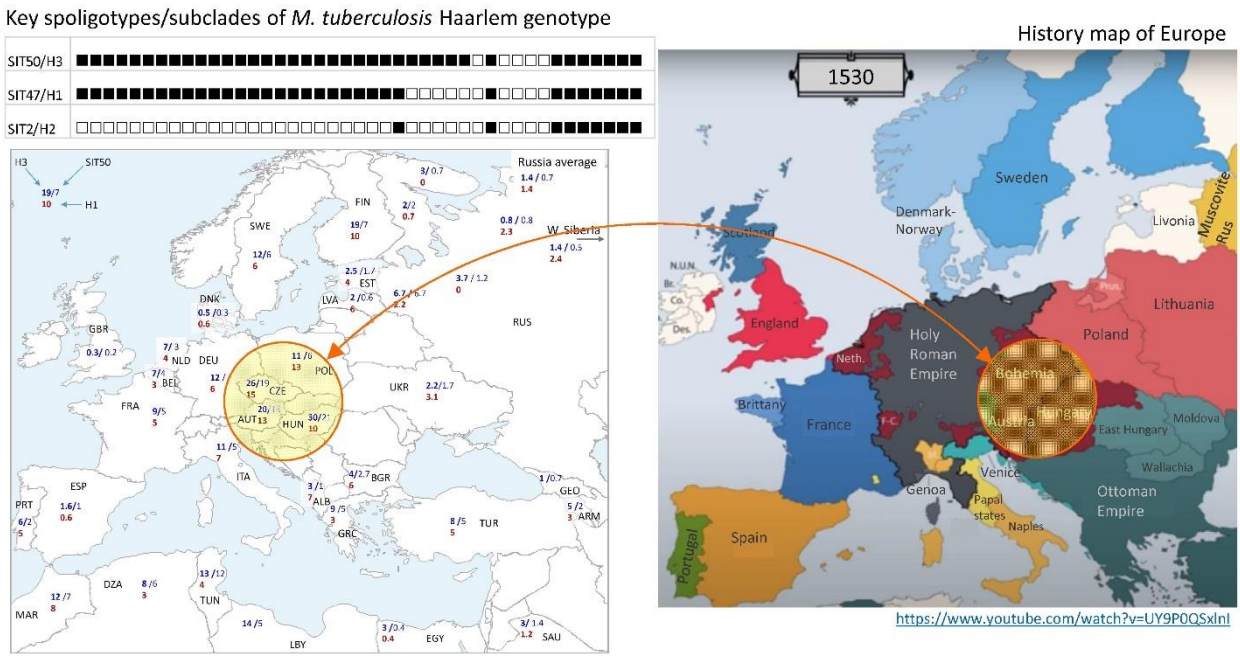

Figure 7. Prevalence of the Haarlem family and is founding spoligotypes in Europe correlated with political map of Europe in XIV century.

Bioarchaeological and molecular evidence of tuberculosis in human skeletal remains from 18th-19th century orthodox cemeteries in Irkutsk, Eastern Siberia

In this study, we tested the skeletal human remains from the 18th - early 19th century Orthodox cemeteries in Irkutsk, Eastern Siberia, for tuberculosis-associated morphological alterations and Mycobacterium tuberculosis DNA. The morphologically studied bone collection included 591 individuals of mainly Caucasian origin. The molecular methods (IS6110-PCR and spoligotyping) suggested that at least four individuals (out of 15 TB-suspected, DNA-tested) were positive for the presence of M. tuberculosis DNA. All of them were males (3 maturus, 1 maturus senilis). Two of them date back to the second and third quarters of the 18th century, another to the last quarter of the 18th century, and the last one to the second half of the 19th century. The combined molecular analysis based on spoligotyping and real-time PCR cautiously suggested presence of different strains and at least some of them represented not the currently predominant in Siberia Beijing genotype (*M. tuberculosis* East-Asian lineage) but strains of European origin. In conclusion, this study presented bioarchaeological and molecular evidence of tuberculosis in human skeletal remains from 18th-19th century Orthodox cemeteries in Irkutsk, Eastern Siberia. The samples are not *M. bovis* and represent human *M. tuberculosis sensu stricto*. Their precise phylogenetic identity is elusive but evokes the European/Russian origin of at least some isolates.

First Insight into Diversity of Minisatellite Loci in *M. bovis*/*M. caprae* in Bulgaria

The aim of this study was to assess the diversity of minisatellite VNTR loci in *Mycobacterium bovis*/*M. caprae* isolates in Bulgaria and view their position within global *M. bovis* diversity. Forty-three *M. bovis*/*M. caprae* isolates from cattle in different farms in Bulgaria were collected in 2015-2021 and typed in 13 VNTR loci. The *M. bovis* and *M. caprae* branches were clearly separated on the VNTR phylogenetic tree. The larger and more geographically dispersed *M.*

caprae group was more diverse than *M. bovis* group was (HGI 0.67 vs. 0.60). Overall, six clusters were identified (from 2 to 19 isolates) and nine orphans (all loci-based HGI 0.79). Locus QUB3232 was the most discriminatory one (HGI 0.64). MIRU4 and MIRU40 were monomorphic, and MIRU26 was almost monomorphic. Four loci (ETRA, ETRB, Mtub21, and MIRU16) discriminated only between *M. bovis* and *M. caprae*. The comparison with published VNTR datasets from 11 countries showed both overall heterogeneity between the settings and predominantly local evolution of the clonal complexes. To conclude, six loci may be recommended for primary genotyping of *M. bovis*/*M. caprae* isolates in Bulgaria: ETRC, QUB11b, QUB11a, QUB26, QUB3232, and MIRU10 (HGI 0.77). VNTR typing based on a limited number of loci appears to be useful for primary bTB surveillance.

PUBLICATIONS

BOOKS, CHAPTERS

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