**Mycobacterium tuberculosis** Population in Northwestern Russia: An Update from Russian-EU/Latvian Border Region

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**Abstract**

This study aimed to characterize the population structure of *Mycobacterium tuberculosis* in Pskov oblast in northwestern Russia, to view it in the geographical context, to compare drug resistance properties across major genetic families. Ninety *M. tuberculosis* strains from tuberculosis (TB) patients, permanent residents in Pskov oblast were subjected to LAM-specific IS6110-PCR and spoligotyping, followed by comparison with SITVITWEB and MIRU-VNTRplus databases. The Beijing genotype (n = 40) was found the most prevalent followed by LAM (n = 18), T (n = 13), Haarlem (n = 10), Ural (n = 5), and Manu2 (n = 1); the family status remained unknown for 3 isolates. The high rate of Beijing genotype and prevalence of LAM family are similar to those in the other Russian settings. A feature specific for *M. tuberculosis* population in Pskov is a relatively higher rate of Haarlem and T types. Beijing strains were further typed with 12-MIRU (followed by comparison with proprietary global database) and 3 hypervariable loci QUB-3232, VNTR-3820, VNTR-4120. The 12-MIRU typing differentiated 40 Beijing strains into 14 types (HGI = 0.82) while two largest types were M2 (223325153533) prevalent throughout former USSR and M11 (223325173533) prevalent in Russia and East Asia. The use of 3 hypervariable loci increased a discrimination of the Beijing strains (18 profiles, HGI = 0.89). Both major families Beijing and LAM had similar rate of MDR strains (62.5 and 55.6%, respectively) that was significantly higher than in other strains (21.9%; P = 0.001 and 0.03, respectively). The rpoB531 mutations were more frequently found in Beijing strains while LAM drug resistant strains mainly harbored rpoBS16 and inhA \(-15\) mutations. Taken together with a high rate of multidrug resistance among Beijing strains from new TB cases (79.3% versus 44.4% in LAM), these findings suggest the critical impact of the Beijing genotype on the current situation with MDR-TB in the Pskov region in northwestern Russia.

**Introduction**

The permanent development of the novel and fine-tuning of the existing molecular approaches permitted a spatio-temporal surveillance of the circulating clones of *Mycobacterium tuberculosis* at global and within-country levels. Association of certain genetic variants of *M. tuberculosis* in Russia with pathogenic properties has been demonstrated in some settings, especially for the Beijing genotype strains although other predominant genotypes deserve no less attention as well. To date, the population of *M. tuberculosis* strains circulating in Russia has been well characterized by different typing methods, although the use of different methodologies across different studies makes it difficult to draw comparable conclusions. Nonetheless, information gained by IS6110-RFLP, 12-MIRU-VNTR and spoligotyping is available in many settings within Russia and in the neighboring countries. Most of the studies carried out by us and others on the vast area of the northwestern Russia focused mainly on St. Petersburg [1,2], Karelia [3], Archangel [4], Murmansk [5] while other provinces received no attention at all or much smaller attention due to inherent limitation of the small sampling per location (e.g., [6]).

The increased human migration results also in active cross-border dissemination of strains hence interest to target such borderline locations. Pskov oblast (670,000 population, 55,400 sq. km surface) is located in northwestern Russia on the Russian-EU/Latvian border. The tuberculosis (TB) incidence here was reported 94.9/100,000 in 2008, 86.7/100,000 in 2009, 82.3/100,000 in 2010 70.8 in 2011 and thus shows a clear trend to decrease; TB prevalence was reported 159.5/100,000 in 2011. At the same time, the rate of drug resistant, and especially, multi-drug resistant TB (MDR-TB) is increasing: MDR-TB was diagnosed in 16.7% of newly diagnosed patients with pulmonary TB in 2011 (V. Krisheiev, unpublished data).

The interest of the present study was to characterize the population structure of *M. tuberculosis* in the Pskov oblast in...
northwestern Russia, to view it in the wide geographical context, and to compare distribution of phenotypic and genotypic drug resistance characteristics across major spoligotype-defined families. Additional analyses/markers were applied to the largest and clinically and epidemiologically important Beijing genotype: (a) 12-MIRU-VNTR loci typing followed by global and regional comparison, (b) use of three hypervariable (HV) VNTR loci to increase a discrimination.

**Materials and Methods**

According to the ethics boards of St. Petersburg Institute of Phthisiopulmonology and St. Petersburg Pasteur Institute, this research does not require ethical approval. The DNA samples were without any personal information about the patients in particular without any ID by name, address, i.e. anonymous samples.

**Study sample**

The 90 studied strains were selected randomly among strains isolated in bacteriology laboratory of Pskov Anti-tuberculosis dispensary from September, 2008 to March, 2009. They included 65 and 25 strains from newly-diagnosed and previously-treated patients, respectively. In particular, 65 strains from newly-diagnosed patients represented 30.6% of all culture-positive new TB cases diagnosed in this period.

Drug susceptibility testing was done for the 1st and 2nd line drugs using recommended MICs by absolute concentration method [7].

**Genotyping**

DNA was extracted using the recommended method [8]. Spoligotyping of isolates was performed as described by Kamerbeek et al. [9]. The spoligoprofiles were entered into Excel spreadsheets and compared with SITVITWEB, an international spoligotype database at Institut Pasteur de Guadeloupe [10].

VNTR (12 MIRU and 3 HV loci) analysis was performed as previously described [11,12]. For allele calling of the HV loci we used a correspondence table kindly provided by Dr. T. Iwamoto.

The MIRU profiles were compared with MIRU-VNTRplus online database [13] in order to verify the lineage determination based on spoligotyping data. In addition, the MIRU profiles of the Beijing genotype strains were compared to the proprietary MIRU-VNTR database of the Beijing genotype [14,15]; at the time of comparison this database included ~2400 strains/profiles from all continents.

Analysis of the specific IS6110 insertion characteristic of the LAM genetic family was done using multiplex PCR as described previously [16].

**Resistance mutations detection**

Mutations in katG315 and inhA promoter region and ahpC associated with INH resistance and mutations in rpoB rifampin resistance determining region (RRDR, codons 507–533) associated with RIF resistance were detected by using TB-Biochip kit (Biochip-IMB, Moscow, Russia) following the manufacturer’s instructions.

**Phylogenetic and statistical analysis**

Hunter Gaston index (HGI) was calculated as described previously [17] and was used to evaluate discriminatory power of the typing schemes.

### Table 1. Spoligotypes of *M. tuberculosis* strains from Pskov area in Russia.

| SIT* | Family | Number of strains | Spoligoprofile |
|------|--------|-------------------|----------------|
| 1    | Beijing| 40                |                |
| 35   | Ural   | 2                 |                |
| 42   | LAM    | 2                 |                |
| 46   | Haarlem| 2                 |                |
| 47   | Haarlem| 2                 |                |
| 50   | Haarlem| 6                 |                |
| 52   | T2     | 1                 |                |
| 53   | T1     | 8                 |                |
| 102  | T      | 3                 |                |
| 252  | LAM    | 8                 |                |
| 254  | LAM    | 3                 |                |
| 262  | Ural   | 3                 |                |
| 266  | LAM    | 2                 |                |
| 267  | LAM    | 1                 |                |
| 444  | LAM    | 2                 |                |
| 1288 | Manu2  | 1                 |                |
| 2021 | T      | 1                 |                |
| 3108 | unknown| 2                 |                |
| ‘new’| unknown| 1                 |                |

*according to SITVITWEB [10].
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Figure 1. Geographic distribution of the main spoligotypes and genetic families identified in *M. tuberculosis* strains in Pskov region and other areas in Russia, former Soviet Union and northern Europe.
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Figure 2. Geographic distribution of the main MIRU types of the Beijing genotypes identified in the Pskov region and other areas of the former Soviet Union and Asia.
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PARS program of the PHYLIP 3.6 package [18] was used to reconstruct the minimum spanning tree of the VNTR digital profiles treated as categorical variables.

A 2 × 2 χ² test was used to detect any significant difference between the two groups. Yates corrected χ² and p-values were calculated with 95% confidence interval using EpiCalc 2000 version 1.02 software [19].

Results and Discussion

Study sample and population structure

The studied collection included 90 M. tuberculosis strains isolated from permanent residents in the Pskov oblast, northwestern Russia, in 2008–2009. No preliminary selection of strains based on their drug resistance or patient status was made. These strains were isolated from patients without proven epidemiological links based on standard contact investigation, 59 males and 31 females, aged from 20 to 73 years old and admitted to the clinics of the Pskov oblast anti-tuberculosis dispensary in 2008–2009. Most of them had infiltrative-pneumonic lung TB (71.1%) and disseminated lung TB (18.8%), according to Russian classification of diseases (both fall within international definition ‘Tuberculosis of lung, confirmed by sputum microscopy with or without culture’, http://apps.who.int/classifications/icd10/browse/2010/en/#/A15.0). Sixty-five (72.2%) patients were newly diagnosed patients who never received anti-TB treatment while 25 (27.8%) patients were previously treated. All enrolled patients were HIV-negative, some of them were former prison inmates (n = 14) and alcoholic (n = 12). These two latter subgroups did not overlap: only one patient was both ex-prisoner and alcoholic. As mentioned above the strains were selected randomly for this study and the high rate of former prison inmates (14/90 [15.5%]) reflect the real situation in the country. In North-West Federal District of Russian Federation (including Pskov oblast), the TB incidence among prisoners was 957/100,000 in 2010. In Russia as a whole, the rate of prisoners among newly-diagnosed TB patients was reported to be 12% in 2009 (T. Otten, personal communication).

Spoligotyping was applied to all strains as a primary typing tool to define the major lineages shaping the general population structure of M. tuberculosis in the Pskov region. Ninety strains were subdivided into 18 spoligotypes. Following comparison with SpolDB4 database they were assigned to the SIT numbers and genetic families (Table 1). In addition, the strains were compared to the MIRU-VNTRplus database and also subjected to the LAM-specific PCR which helped us to clarify/change the family status of some spoligotypes. In particular, SIT254, SIT444 (T5_RUS1) SIT266, SIT267 (T family) were redefined as LAM; SIT35 and SIT262 (Haarlem4) were redefined as Ural. Consequently, this changed a relative proportion of strains belonging to certain genetic families in our collection. In total, the Beijing genotype (40/90) was found the most prevalent in our setting followed by LAM (n = 18), T superfamily (n = 13), Haarlem (n = 10), Ural

![Figure 3. Minimum spanning tree of the 12-MIRU-VNTR profiles of the Beijing strains from the Pskov region in the northwestern Russia. 12-loci digital profile is shown in the 1st line at the each node, alleles of 3 HV loci (VNTR-4120, VNTR-3820, QUB-3232) are shown in the 2nd line. Locus number and allele change are shown on branches. Circle size is roughly proportional to the number of strains. doi:10.1371/journal.pone.0041318.g003](http://apps.who.int/classifications/icd10/browse/2010/en/#/A15.0)
The family status remained unknown for 3 isolates after comparison with SITVITWEB international database [10].

The history of alcoholism and imprisonment were not significantly associated with infection with particular strain genotype in this study. LAM family was the most prevalent among alcoholics (n = 5) followed by Beijing (n = 3), T (n = 3) and Haarlem (n = 1). Interestingly, all 3 Beijing strains and only 1 of 5 LAM strains were from newly-diagnosed patients. On the other hand, all three Beijing strains and 4 of 5 LAM strains were drug resistant.

The 14 strains isolated from ex-prisoners belonged to the following families: Beijing (n = 7), Haarlem (n = 3), LAM (n = 1), T (n = 1), Ural (n = 1), unknown (n = 1). When contrasting ex-prisoners and patients without prison history, the Beijing genotype constituted similar proportions in both groups: 7 (50.0%) of 14 and 34 (44.7%) of 76, respectively. In contrast, LAM strains were isolated from 1 (7.1%) of 14 ex-prisoners and 17 (22.4%) of 76 other patients, respectively (P = 0.3). Regarding drug resistance of ‘prison’ strains, all 7 Beijing strains and only 1 of 7 non-Beijing strains were drug-resistant. Previous studies in the former Soviet Union found a higher rate of Beijing genotype in ex-prisoners [20,21,22]. An exception was Central Russian study that highlighted an important prevalence of the LAM family (44.8%) similar to that of the Beijing genotype (43.7%) [23]. Summing up, a better transmission of Beijing strains under overcrowded

### Table 2. Global distribution of the MIRU profiles identified in *M. tuberculosis* Beijing genotype strains from Pskov.

| Type, % in database (if >0.5%)* | 12-MIRU-loci profile | Pskov, number (%) | Former Soviet Union, area and % | Mainland China, area and % | East Asia other than China, country and % |
|--------------------------------|----------------------|------------------|---------------------------------|--------------------------|------------------------------------------|
| M2 (14.8)                      | 223325153533         | 13 (32.5)        | Northwest 53, Samara 58, Kaliningrad 17, Ural 24, Irktusk 26, Kaliningrad 65, South Ukraine 26, Kyrgyzstan 73 | Beijing 2.7, Shanghai 2.7, Wuhan 3.6, Henan 6.2; Japan 0.5 |
| M11 (25.4)                     | 223325173533         | 11 (27.5)        | Northwest 21, Samara 24, Ural 56, Irktusk 26, Kaliningrad 65, South Ukraine 26, Kyrgyzstan 2.6 | Beijing 46, Shanghai 12, Wuhan 13, Henan 19; Japan 24, Laos 28, Mongolia 66, Vietnam 21 |
| M8                             | 22325173533          | 2 (5)            | Northwest 2.1, Ural 2            | Beijing 2.7, Wuhan 17, Henan 3.1; Japan 0.3 |
| M40 (0.5)                      | 223325153523         | 1 (2.5)          | Kaliningrad 2.5, Irktusk 3.5, Kyrgyzstan 2.6 | Shanghai 1.3 |
| M9                             | 223351735331         | 2 (5)            | Northwest 2.1, Kaliningrad 2.5   | Beijing 2.7, Shanghai 1.3, Henan 1.3; Mongolia 11, Vietnam 1.9 |
| M12                            | 221351735333         | 1 (2.5)          | Northwest 2.1, Samara 1.6, Ural 6, Kaliningrad 2.5 | Beijing 1.3, Shanghai 1.3, Henan 6.2; Japan 13 |
| M39                            | 222351535333         | 1 (2.5)          | Kaliningrad 5                     | Japan 0.3, Vietnam 1.9 |
| M41                            | 224351735333         | 1 (2.5)          | Kaliningrad 5                     | Japan 0.3 |
| M89                            | 22335153534          | 3 (7.5)          | Ural 2, Irktusk 0.9               | |
| M112                           | 223351935335         | 1 (2.5)          |                                 | Beijing 1.3 |
| M216                           | 2225151534           | 1 (2.5)          |                                 | |
| M219                           | 22341735333          | 1 (2.5)          |                                 | |
| M224                           | 22335154533          | 1 (2.5)          |                                 | |
| M231                           | 22336153533          | 1 (2.5)          |                                 | |

*according to the updated version (2400 strains) of the MIRU global database of Beijing genotype [14,15] updated with more recent raw data on Henan, Mongolia, Japan-Osaka [44], Laos [45], Ukraine [32], Kaliningrad [30], Kyrgyzstan [21].

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### Table 3. Discriminatory capacity of different VNTR typing schemes applied to the Beijing genotype strains from the Pskov region in the northwestern Russia.

| Characteristic | 12-MIRU loci | +3 HV* | +3232 | +3820 | +4120 | +3232 and +3820 | +3232 and +4120 | +3820 and +4120 |
|----------------|--------------|--------|-------|-------|-------|-----------------|-----------------|-----------------|
| Cluster** range| 2–13         | 2–10   | 2–10  | 2–11  | 2–10  | 2–10           | 2–10           | 2–10           |
| Number of clusters | 5           | 5      | 5     | 5     | 6     | 5               | 5               | 5               |
| Clustered       | 31           | 26     | 27    | 27    | 29    | 26              | 27              | 26              |
| Singletons      | 9            | 14     | 13    | 13    | 11    | 14              | 13              | 14              |
| HGI            | 0.823        | 0.896  | 0.886 | 0.873 | 0.885 | 0.896           | 0.886           | 0.896          |

*Three hypervariable (HV) loci, namely, QUB3232, VNTR3820, VNTR4120.

**Cluster is defined as a group of strains with identical multi-locus digital signature.

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conditions in a prison environment has been demonstrated in most settings but not in our study.

To view the distribution of the families identified here beyond its target area, we compared the spoligotype based population structures of *M. tuberculosis* in Pskov region and other areas of the former USSR as well as Poland and Finland [2,3,14,20,21,24,25,26,27,28,29,30,31,32,33,34,35,36,37]; the pie charts are shown in Fig. 1. It is clear and otherwise well-known that Beijing genotype is relatively or absolutely prevalent throughout all countries of the former Soviet Union but found in extremely low rate in Finland and Poland. Sinkov et al. [38] have recently suggested an intriguing hypothesis about primary penetration of the Beijing genotype from China to the Soviet Union only in the 20th century but not earlier. They reasoned that

### Table 4. Drug susceptibility and prevalence of drug resistance mutations in *M. tuberculosis* strains of Beijing, LAM and other genotypes isolated from TB patients in Pskov region of Russia.

| Phenotype*, genotype | All strains, n = 90 | Beijing genotype, n = 40 | LAM genotype, n = 18 | Other genotypes, n = 32 | Beijing M2, n = 13 | Beijing M11, n = 11 |
|----------------------|---------------------|--------------------------|----------------------|------------------------|------------------|-------------------|
| Fully susceptible    | 34                  | 5                        | 5                    | 24                     | 0                | 0                 |
| Rif-resistant        | 43                  | 26                       | 10                   | 7                      | 12               | 9                 |
| INH-resistant        | 50                  | 30                       | 13                   | 7                      | 13               | 10                |
| STR-resistant        | 55                  | 34                       | 13                   | 8                      | 13               | 11                |
| EMB-resistant        | 33                  | 17                       | 10                   | 6                      | 10               | 4                 |
| PZA-resistant        | 6                   | 3                        | 2                    | 1                      | 2                | 1                 |
| OFL-resistant        | 6                   | 4                        | 2                    | -                      | 1                | 1                 |
| ETH-resistant        | 12                  | 3                        | 8                    | 1                      | 1                | -                 |
| KAN-resistant        | 16                  | 5                        | 7                    | 4                      | 3                | 1                 |
| CAP-resistant        | 11                  | 2                        | 6                    | 3                      | -                | -                 |
| MDR                  | 42                  | 25                       | 10                   | 7                      | 12               | 9                 |
| XDR                  | 5                   | 3                        | 2                    | -                      | 1                | -                 |
| rpoB533              | 24                  | 19                       | -                    | 5                      | 10               | 6                 |
| rpoB526              | 2                   | 1                        | 1                    | -                      | 1                | 1                 |
| rpoB516              | 8                   | -                        | 8                    | -                      | -                | -                 |
| rpoB507              | 1                   | -                        | -                    | 1                      | -                | -                 |
| rpoB512              | 2                   | 1                        | 1                    | -                      | -                | -                 |
| rpoB wild type       | 52                  | 19                       | 8                    | 25                     | 2                | 4                 |
| katG 315-ACC         | 44                  | 26                       | 12                   | 6                      | 12               | 8                 |
| inhA-15T             | 10                  | -                        | -                    | -                      | -                | -                 |
| katG 315-ACC & inhA-15T | 10               | -                        | -                    | -                      | -                | -                 |

*RIF, rifampin; INH, isoniazid; STR, streptomycin; EMB, ethambutol; PZA, pyrazinamide; OFL, ofloxacin; ETH, ethionamide; KAN, kanamycin; CAP, capreomycin; MDR, multidrug-resistant; XDR, extremely drug resistant.

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### Table 5. Drug susceptibility of *M. tuberculosis* strains of different genotypes from newly-diagnosed and previously-treated patients.

| Characteristic            | Newly-diagnosed patients (n = 65), number, % | Drug-resistant strains of the respective genotype, number, % | Previously-treated patients (n = 25), number, % |
|---------------------------|---------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|
| Genotype                  |                                             |                                                               |                                               |
| Beijing                   | 29, 44.6                                    | 23, 79.3                                                      | 11, 44.0                                      |
| LAM                       | 9, 13.8                                     | 4, 44.4                                                       | 9, 36.0                                       |
| Haarlem                   | 7, 10.8                                     | 1, 14.3                                                       | 2, 8.0                                        |
| T                         | 12, 18.5                                    | 1, 8.3                                                        | 1, 4.0                                        |
| Ural                      | 4, 6.2                                      | 2, 50.0                                                       | 1, 4.0                                        |
| Phenotype                 |                                             |                                                               |                                               |
| Drug-resistant            | 31, 47.7                                    | 24, 96.0                                                      |                                               |
| Drug-susceptible          | 34, 52.3                                    | 1, 4.0                                                        |                                               |

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Finland was a part of the Russian Empire and since Beijing is not endemic in Finland, apparently it should have arrived to Russia after Finland's independence in 1918. However one should keep in mind that although TB mortality was very high in Russia early 20th century (~400,000,000 [39]), the country remained agrarian and not densely populated. Finland became a part of the Russian Empire only in 1810, furthermore it kept its semi-autonomous status and the Russian presence was limited to the civil servants and military garrisons and constituted only few percent of the total population of Finland (http://en.wikipedia.org/wiki/Russians_in_Finland). In this view, the Beijing strain would hardly be disseminated by Russians throughout Finland in any case.

Regarding countries of the former Soviet Union, in spite of the very high rate of the Beijing genotype in all settings, one may note some gradient of its prevalence with higher rate in Central Asia (Kazakhstan and Kyrgyzstan, >60%) versus relatively lower rate (mostly 40% or less) in the western borderline areas of the former USSR from Georgia in south to Pskov and Karelia in north (e.g., P = 0.01 for Pskov versus Kazakhstan comparison, Fig. 1).

The 2nd important family in this study was LAM (20%). This family is also found in other areas of the European part of the former Soviet Union, e.g. Kharkov in South Ukraine (23% [26]), Kaliningrad (18% [30]), but in lesser rate in Ural and west Siberia (9-10% [31,40]) although a more recent study in Novosibirsk in west Siberia described 18% of LAM [25]. In spite of the phylogeographically meaningful name (Latin American Mediterranean), at present this family is globally distributed and its within-Russian gradient may be observed only on the large scale when comparing European versus Asian parts of Russia and former Soviet Union as a whole.

A feature specific for Pskov is a relatively higher rate of Haarlem and T types which is similar to Poland and Finland but quite different from other Russian regions. This is well in line with westernmost location of Pskov region within mainland Russia.

It is also interesting to note some gradient of the less known genetic family Ural in the south-eastern area on the map. It was recently suggested that central Eurasia may be an area of primary dissemination of this family, north/north-east Pontic area would be its origin [41] while more distant areas of northwestern (including Pskov) and central Russia exhibit a negligible rate of the Ural family (Fig. 1).

Structure of the Beijing genotype subpopulation

We further analysed the population structure of the Beijing family that was predominant in this study and is known to be epidemiologically and clinically important genotype of M. tuberculosis [10,15,22,42,43,44,45]. To this end, we used 12-MIRU-VNTR typing followed by comparison with international database of the Beijing genotype [14,15]. We did not aim to achieve a high discrimination of single strains but rather to identify the major MIRU-types and compare their distribution in Pskov region against neighboring and more distant areas in Eurasia.

The 12-MIRU typing differentiated 40 Beijing strains into 14 types while two types M2 (223325153533) and M11 (223325173533) were found in similar and high rates (Table 2); for reference and comparability purposes it may be noted that these types correspond to MIT16 and MIT17, respectively, in the M. tuberculosis Typing in Northwestern Russia

Drug resistance properties: genotype and phenotype

The drug susceptibility testing of the 1st and 2nd line anti-TB drugs and detection of the genetic determinants of drug resistance to the major drugs rifampin (RIF) and isoniazid (INH) was done for all strains. The results are shown in Table 4. We also compared distribution of drug resistance in major genetic families Beijing (and its subtypes M2 and M11) and LAM.

Thirty-four strains were susceptible while 56 strains were found resistant to at least one drug; of these latter 42 strains were MDR and 5 strains were XDR. Regarding strains from newly-diagnosed patients, 34 were susceptible, 31 drug-resistant, 18 MDR, 1 XDR. The level of mono-resistance generally reflects the quality of treatment and compliance. In this study, five strains were resistant to a single drug (streptomycin [STR]), while 4 of them were Beijing genotype and one strain belonged to T family (SIT53). STR is known to have been widely used in the 1990s in Russia and almost all Russian drug resistant strains regardless of their
genotype are resistant to at least STR. Thus this finding of STR monoresistant strains is not surprising. However absence of RIF and INH monoresistant strains may be considered as an indirect evidence of sufficient level of compliance of patients infected with drug susceptible strains in Pskov.

It should be noted that both major families Beijing and LAM had similar rate of MDR strains: 25 of 40 Beijing versus 10 of 18 LAM strains were MDR (P = 0.83). On the other hand, both included a higher percent of MDR strains when compared to other genotypes pooled together. Twenty-five of 40 Beijing versus 7 of 32 other (non-Beijing, non-LAM) strains (P = 0.001 5.95 [2.07; 17.09], and 10 of 18 LAM versus 7 of 32 other (non-Beijing, non-LAM) strains (P = 0.03, OR 0.54 95% CI [0.30; 0.99]) were MDR. A recent Ukrainian study showed an association of MDR with LAM but not Beijing genotype [26], i.e. 11 of 16 LAM and 17 of 31 Beijing were MDR. Even if a small sample size may be a reason, this result suggests (as in our study) a general trend of the family/phenotype association in a high TB-burden area: the drug resistance is ultimately associated with all major circulating families. In case of Russia these are Beijing and LAM. This finding highlights an importance to consider not only the notorious Beijing genotype but also other circulating families in an area, in particular, to compare not only the Beijing genotype versus all other families taken together but perform more detailed comparisons.

On the other hand, one should note an interesting distribution of the particular mutations/codons in rpoB in different families. For example, rpoB531 mutations were more frequently found in Beijing strains while LAM RIF resistant strains usually had rpoB5316 mutations. A similar finding of the rpoB5316 mutations found mainly in LAM strains was published by Ignatova et al. [23]. Different mechanisms related to the second order selection [50] and/or compensatory mutations in other genes [51] may be hypothetically related to this bias. On the other hand, the quality of the drug used in different countries may also hypothetically contribute to the preferential selection of some mutations.

An association of rpoB531 mutation with Beijing genotype was shown in many other studies carried out in the former Soviet Union [22,24,25,30,32]. On the other hand, the very high rate of this (otherwise relatively most frequent) mutation in Beijing strains is not inevitable feature of this genotype only. For example, a study in Bulgaria found high rate of this mutation in the RIF-resistant strains of different (non-Beijing) genotypes [52]. The katG315 AGC>ACC mutation was frequently described in INH resistant strains, especially in the high TB-burden areas [22,25,30,32]. Thus its finding in the present study is not unexpected: katG315 AGC>ACC was found in similar rate in 26 of 30 Beijing and 12 of 13 LAM, and 6 of 7 other genotypes’ INH-resistant strains. An intriguing feature that is also observed in Table 4 is a clear difference in prevalence of inhA -15C>T promoter mutations. First, all 10 strains with -15C>T mutation harbored also katG315 mutation; this is not unusual since these

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\text{inhA} \text{ mutations are known to provide secondary mechanism of INH resistance. Second, and more remarkable is that these 10 strains were all and only different types of the LAM family. One should note that this was not observed in the Moscow study where inhA promoter mutations were found in different genotypes [24].}
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In terms of resistance properties within Beijing subtypes (MIRU types), neither M2 nor M11 included fully susceptible strains, and almost completely consisted of MDR strains. Multidrug resistance was frequent among Beijing M2 and M11 types (87.5%) than in other Beijing MIRU types taken together (12.5%) (P < 0.0001). Interestingly all drug susceptible Beijing strains belonged to the minor types M8, M39, M41, M89, M219 h M231.

We looked more closely at the subsample of our collection representing strains isolated form newly-diagnosed patients (Table 5). The Beijing strains were found in similar rate in both newly-diagnosed and previously-treated TB subsamples (44.6 and 44.0%, respectively). In contrast, LAM strains were more visible among previously-treated patients (56.0 vs 13.9%, P = 0.04 OR 3.50 95% CI [1.19; 10.29]). Taken together with a high rate of drug resistance among Beijing strains from new TB cases, these findings suggest the critical impact of the Beijing genotype on the current situation with TB in this region in northwestern Russia.

Concluding remarks

The M. tuberculosis population in Pskov region in northwestern Russia features all major genetic families characteristic for Russia and its European part, the Beijing and LAM genotypes being the most prevalent. In contrast to other Russian regions, M. tuberculosis population in Pskov is marked with a relatively higher proportion of Haarlem and T types.

The 12-MIRU typing differentiated 40 Beijing strains into 14 types while two largest types were M2 (2332351533533) prevalent throughout Russia and ex-USSR and M11 (2332351733533) prevalent in Russia and East Asia. Whereas 12-MIRU identified 14 profiles (HGI = 0.82), the use of 3 HV loci improved discrimination resulting in 18 profiles (HGI = 0.89).

Comparison with drug resistance data suggests a general trend of the family/phenotype association in a high TB-burden area: the multidrug resistance is associated with all major circulating families. In case of Russia these are Beijing and LAM. This highlights an importance to monitor not only the notorious Beijing genotype but also other M. tuberculosis families in an area.

The prevalence of the Beijing genotype along with high rate of drug resistance among Beijing strains from new TB cases suggest the critical impact of the Beijing genotype on the ongoing transmission of MDR-TB in this region in northwestern Russia.

Author Contributions

Conceived and designed the experiments: IM VK BV ON. Performed the experiments: IM AV VK BV ON. Wrote the paper: IM AV VK BV ON.

References

1. Narvskaya OV, Mokrousov IV, Otten TF, Vishnevskii BI (1999) Genetic marking of polyresistant Mycobacterium tuberculosis strains isolated in the northwest of Russia. Probl Tuberk (3): 39–41. In Russian.
2. Narvskaya O, Mokrousov I, Otten T, Vishnevskii B (2005) Molecular markers: application for studies of Mycobacterium tuberculosis population in Russia. In: Read MM, editor. Trends in DNA fingerprinting research. New York, USA: Nova Science Publishers. pp. 111–125.
3. Markelov Y, Narvskaya O (2010) Circulation of multidrug-resistant tuberculous pathogen strains in the Republic of Karelia. Tuberk Bolezn Legk (2): 54–56. In Russian.
4. Toungoussova OS, Sandven P, Mariandyshov AO, Nizovtseva NI, Bjune G, et al. (2002) Spread of drug-resistant Mycobacterium tuberculosis strains from the Beijing genotype in the Archangel Oblast, Russia. J Clin Microbiol 40: 1930–1937.
5. Makinen J, Jarjama M, Haapenra-Heikkinen M, Marttila H, Endourova LB, et al. (2011) Extremely high prevalence of multidrug resistant tuberculosis in Murmansk, Russia: a population-based study. Eur J Clin Microbiol Infect Dis 30: 1119–1126.
6. Baranov AA, Mariandyshov AO, Mannaker T, Dahlé UR, Bjune GA (2009) Molecular epidemiology and drug resistance of widespread genotypes of Mycobacterium tuberculosis in northwestern Russia. Int J Tuberc Lung Dis 13: 1208–1295.
7. Ministry of Health of Russian Federation (2003) Order No 109 “On improvement of anti-tuberculosis activities in Russian Federation”. Moscow.
8. van Embden JDA, Cave MD, Crawford JT, Dale J RV, Eisenach KD, et al. (1993) Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 31: 406–409.
9. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soelen D, et al. (1997) Simultaneous detection and strain differentiation of Mycobacterium tuberculosis complex by DNA fingerprinting. J Clin Microbiol 35: 907–914.
10. Denay C, Liens B, Bourgureau T, Hill V, Couvin D, et al. (2012) SEVTIWEB—A publicly available international multilocus database for studying Mycobacterium tuberculosis genetic diversity and molecular epidemiology. Infect Genet Evol 12: 757–766.
11. Supply P, Lesjean S, Savin E, Kremer K, van Soolingen D, et al. (2001) Iwamoto T, Yoshida S, Suzuki K, Tomita M, Fujiyama R, et al. (2007) Molecular analysis of Mycobacterium tuberculosis strains isolated in Ural region, Russian Federation, by MIRU-VNTR genotyping. Int J Tuberc Lung Dis 9: 746–752.
12. Supply P, Lesjean S, Savin E, Kremer K, van Soolingen D, et al. (2001) Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. J Clin Microbiol 39: 3563–3571.
13. Iwamoto T, Yoshida S, Suzuki K, Tomita M, Fujiyama R, et al. (2007) Hypervariable loci that enhance the discriminatory ability of newly proposed 15-loci and 24-loci variable-number tandem repeat typing method on Mycobacterium tuberculosis strains predominated by the Beijing family. FEMS Microbiol Lett 282: 283–282.
14. Niemann S, Diel R, Khechinashvili G, Gegia M, Mdivani N, et al. (2010) Felsenstein J (2004) PHYLIP (Phylogeny Inference Package) version 3.6b. Seattle.
15. Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, et al. (2006) Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, et al. (2006) Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape Province of South Africa. J Clin Microbiol 44: 3339–3343.
16. Vasilenko N, Vyazovaya A, Mokrousov I, Limeschenko E, Semenov V, et al. (2006) Genetic variation of Mycobacterium tuberculosis isolates in patients newly diagnosed with tuberculosis in the Novosibirsk oblast, Russia. J Med Microbiol 55: 1413–1418.
17. Dymova MA, Khinto VN, Chernevichko AG, Khrapov EA, Swierulik AV, et al. (2011) Highest prevalence of the Mycobacterium tuberculosis Beijing genotype isolates in patients newly diagnosed with tuberculosis in the Novosibirsk oblast, Russian Federation. J Med Microbiol 60: 1003–1009.
18. Dymova MA, Liashchenko OP, Potekhi PI, Krutko VS, Khrapov EA, et al. (2011) Genetic variation of Mycobacterium tuberculosis circulating in Kharkov Oblast, Ukraine. BMC Infect Dis 11: 77. Available: http://www.biomedcentral.com/1471-2334/11/77. Accessed 27 June 2012.
19. Väänänen N, Vazyova A, Mokrousov I, Länsenaho E, Semenov V, et al. (2006) Spacre oligonucleotide typing of drug-resistant Mycobacterium tuberculosis circulating on the territory of Belarus. Immunomut Allerged Infekt (4): 70–74. In Russian.
20. Ignatova A, Dubiley S, Stepanshina V, Shemyakin I (2006) Predominance of Beijing strain family in Russia. JAMA 293: 2726–2731.
21. Drobniewski F, Balabanova Y, Nikolayevskyy VV, Bazhora YI, Asmolov AA, Balabanova YM, et al. (2005) Beijing strain family in East Asia revealed through refined population structure analysis. FEMS Microbiol Lett 291: 35–43.
22. Ignatova A, Dubiley S, Stepanshina V, Shemyakin I (2006) Beijing strain family in the 20th century. Epidemiologiya i infekciinnoye bolezni (4): 50–53. In Russian.
23. Manev A, Brzostek A, Poplawska A, Rastogi N, Sola G, et al. (2004) Molecular epidemiology of drug-resistant Mycobacterium tuberculosis strains isolated from patients with pulmonary tuberculosis in Poland: a 1-year study. Int J Tuberc Lung Dis 8: 1448–1453.
24. Drobniewski F, Balabanova Y, Nikolayevskyy VV, Bazhora YI, Asmolov AA, Balabanova YM, et al. (2005) Beijing strain family in East Asia revealed through refined population structure analysis. FEMS Microbiol Lett 291: 35–43.
25. Balabanova Y, Nikolayevskyy VV, Brown TJ, Bazhora YI, Asmolov AA, Balabanova YM, et al. (2005) Beijing strain family in East Asia revealed through refined population structure analysis. FEMS Microbiol Lett 291: 35–43.
26. Sajduda A, Brzostek A, Poplawska A, Rastogi N, Sola G, et al. (2004) Molecular epidemiology of drug-resistant Mycobacterium tuberculosis strains isolated from patients with pulmonary tuberculosis in Poland: a 1-year study. Int J Tuberc Lung Dis 8: 1448–1453.
27. Manneh S, Miller V, Lofthus P, Soini H (2008) Molecular genetics of drug-resistant Mycobacterium tuberculosis isolates in Finland, 1995–2004. Int J Tuberc Lung Dis 12: 338–343.
28. Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, et al. (2006) Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape Province of South Africa. J Clin Microbiol 44: 3339–3343.
29. Ignatova A, Dubiley S, Stepanshina V, Shemyakin I (2006) Predominance of Beijing strain family in Russia. JAMA 293: 2726–2731.
30. Mokrousov I, Otten T, Zucco T, Turkin E, Nazemtseva V, et al. (2009) At Baltic crossroads: a molecular snapshot of Mycobacterium tuberculosis population diversity in Kaliningrad, Russia. FEMS Microbiol Immunol 55: 13–22.
31. Wojtylowicz A, Schluter H, Heinrich A, Rastogi N, Marques M, et al. (2010) Molecular epidemiology of drug-resistant Mycobacterium tuberculosis strains circulating in Bulgaria. J Clin Microbiol 48: 388–390.
32. Rossini A, Ignatova A, Dubilevsky S, Poplawska A, Rastogi N, Sola G, et al. (2004) Molecular epidemiology of drug-resistant Mycobacterium tuberculosis strains isolated from patients with pulmonary tuberculosis in Poland: a 1-year study. Int J Tuberc Lung Dis 8: 1448–1453.
33. Tracevska T, Jansone I, Baumanis V, Sola G, Lillebaek T (2003) Prevalence of Beijing genotype in Latvian multidrug-resistant Mycobacterium tuberculosis isolates. J Clin Microbiol 41: 1521–1523.
34. Mokrousov I, Otten T, Zucco T, Turkin E, Nazemtseva V, et al. (2009) At Baltic crossroads: a molecular snapshot of Mycobacterium tuberculosis population diversity in Kaliningrad, Russia. FEMS Microbiol Immunol 55: 13–22.
35. Conner T, Essioux E, Sillust R, Danilovits M, Levina K, et al. (2001) Spread of drug-resistant pulmonary tuberculosis in Estonia. J Clin Microbiol 39: 3339–3343.