Analysis of Mycobacterium tuberculosis genetic lineages circulating in Riga and Riga region, Latvia, isolated between 2008 and 2012

Ilva Pole, Julija Trofimova, Inga Norvaisa, Philip Supply, Girts Skenders, Anda Nodieva, Iveta Ozere, Vija Riekstina, Viktorija Igumnova, Jelena Storozenko, et al.

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Title: Analysis of Mycobacterium tuberculosis genetic lineages circulating in Riga and Riga region, Latvia, isolated between 2008 and 2012.

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Abstract: Although the number of new tuberculosis (TB) cases registered per year has decreased by 3-fold between 2001 and 2017 in Latvia, the TB incidence and rates of multidrug resistant TB in this Baltic country remain substantially higher than in most other European countries. Molecular typing methods of Mycobacterium tuberculosis (MTB) play an important role both in clinical studies of the disease and the epidemiological investigations, allowing to describe and characterize the pathogen's population structure and spread of particular genotypes. Aim of this study was to examine the prevalence of MTB lineages in Riga and Riga region of Latvia within a five-year period (2008 - 2012), and to evaluate the discriminatory power (DP) of spoligotyping, standard 24-locus MIRU-VNTR and IS6110-RFLP methods in this setting. The results showed that the main MTB spoligotype families were Beijing (25.3%) and LAM (24.3%), followed by T (22.1%), Ural (11.2%), Haarlem (6.6%) and X superfamily (3.4%). This distribution remained stable over the five consecutive years. 67.6% of MTB isolates were pan-susceptible, and 32.4% were resistant to any drug; multi-drug resistance was found in 5.8% of MTB strains, and 7.6% of MTB isolates were extensively drug-resistant. Drug resistance was associated with SIT1, SIT283 and SIT42 genotypes, while SIT1 and SIT42 were overrepresented among multi drug-resistant MTB strains. Overall, DP of spoligotyping method alone was 0.8953, while DP of both 24-locus MIRU-VNTR analysis and IS6110 RFLP was higher (DP=0.9846 and 0.9927, respectively), mainly due to the improvement of the resolution for the Beijing strains. In conclusion, this work represents the first comprehensive molecular epidemiological description of TB in Latvia, highlighting the high genetic diversity of MTB strains circulating in Riga and Riga region. In combination with detailed epidemiological data this approach was helpful for the in-depth understanding of epidemiological processes in settings where the Next-Gen sequencing is not available as a routine method.

June 21, 2019

Editor-in-Chief

Michel Tibayrenc

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(MEEGID)

Enclosed, please find our manuscript entitled „*Analysis of Mycobacterium tuberculosis genetic lineages circulating in Riga and Riga region, Latvia, isolated between 2008 and 2012*”.

This article is submitted to the Special issue on St. Petersburg Symposium on TB & Mycobacteria (Edited by Igor Mokrousov).

It was preliminarily discussed with Dr Igor Mokrousov who encouraged this submission and thus may serve as its handling editor.

Tuberculosis is one of the major infectious diseases in Latvia, a country in the Baltic region of the northeastern Europe. Molecular genotyping methods of *Mycobacterium tuberculosis* play an important role both in clinical studies of the disease and in the epidemiological investigations, allowing to describe and characterize pathogen`s population structure. The aim of this study was to examine the prevalence of *M. tuberculosis* lineages in Riga and Riga region of Latvia within the five year period (2008 – 2012), and to evaluate the discrimination power of spoligotyping, MIRU-VNTR and IS6110-RFLP methods for genotyping of *M. tuberculosis* clinical isolates in this setting.

This report for the first time describes the molecular epidemiology of tuberculosis in Latvia.

The corresponding author of the manuscript, Renate Ranka, is a senior researcher in Latvian Biomedical Research and Study Centre.

I certify that this manuscript, or any part of it, has not been published and will not be submitted elsewhere for publication while being considered by the MEEGID.

No competing interests are present.

Sincerely yours,

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Response to Reviewers.

We thank the Editor and the reviewers for constructive comments that we feel substantially improved the manuscript. We have done our best to change the manuscript according to the recommendations.

Please note, that the numbering of tables and supplementary tables was changed as new data were added to the manuscript.

Reviewers' comments:

Reviewer #1: Very good and sound paper. There will be one of the significant epidemiological works, with an excellent sampling.

My comments are below.

1. It is a pity that there is no information on drug sensitivity, although this would undoubtedly strengthen the article and shed light on the epidemiological significance of the identified genotypes (spoligotypes, clusters, RFLP types).

Moreover, the introduction gives such a characteristic: "Latvia is among the multidrug-resistant tuberculosis (MDR-TB) middle burden countries in the world: there were 36 (7.5%) notified. MDR TB cases in 2017 (data from The Center for Disease Prevention and Control of Latvia)."

and the discussion mentions the association of the Beijing genotype with resistance, including reference to earlier studies in Latvia ("In 281 Latvia, too, Beijing genotype was prevalent among MDR-TB patients (Tracevska et al., 2003)."

Thus a comparison would allow to find out what proportion in this study are MDR strains, of which genotypes, clusters, and dynamics can be traced from 2008 to 2012.

Our response.

The information on drug resistance for the studied isolates was added (Table 4).

Sections of the manuscript (Abstract, Materials and Methods, Results, Discussion) were changed accordingly.

2. The article does not discuss clusters 94-32 and 100-32 at all. It is interesting if there are any changes in their proportions over the years.

Our response.

The information in Discussion section was added.

However, it was not possible to track the changes of the proportions of these MIRU-VNTR clusters over the years, as this analysis was not performed for all MTB isolates (please see also the answer below).

3. It would also be interesting to know about SIT262 (that "was associated with imprisonment"): how similar were VNTR profiles and DST profiles? Also, take note of recent paper of Sinkov et al. BMC Genomics 2018 about different association with DR of the Ural spoligotypes.

Our response.

Again, unfortunately, MIRU-VNTR was not available for all samples. The obtained data showed that MIRU/VNTR clustering rate for Ural family was not high (17 out of 46 isolates in 5 clusters); only two clusters (4 and 3 isolates) for SIT262 (Suppl. Table 3).

The drug susceptibility testing revealed that 22 of 23 SIT262 isolates were pan-susceptible (Table 4).

The information in the Discussion section was added.

4. The above questions also concern SIT254 LAM, in particular in terms of drug resistance.

Our response.

The information was added (Table 4). The majority of samples were drug-susceptible.

5. I noticed that the studied isolates were also obtained from children (43 aged below 20 years, in table 1). What % of all TB-diagnosed children during this period. Any idea about family contacts? Indeed this may be a subject of a separate article.

Our response:

The information was added in the Results section.

In total, within the youngest age group 16 MTB isolates were from children; no infectious source case was identified for any of these cases. All other culture-positive pediatric cases for whom epidemiological links were identified were included in a separate study (this study is in progress).

6. It would be interesting to track the dynamics of the number of clusters (VNTR) in different genotypes. Did it change or remained stable? Or at least make a table by years of main spoligotypes, to see if the structure changed.

Our response:

The authors agree that the dynamics of VNTR clusters could be interesting to explore. Unfortunately, in this study, the VNTR analysis was not available for all isolates. Only 411 samples, which were obtained mainly in years 2009-2010, were studied to explore the DP of genotyping methods in our settings. As the distribution of these samples within the years is not uniform, the analysis of the dynamics will not be accurate.

The prevalence of main spoligotypes (overall prevalence >1%) by years is presented in new Supplementary Table S2.

Highlights.

- Molecular epidemiology of tuberculosis in Latvia was described for the first time
- High diversity of *M. tuberculosis* strains was found
- Drug resistance of *M. tuberculosis* isolates was analyzed.
- Association between spoligotypes and TB patients` age was found
- Combination of genotyping methods increased the resolution of the genotypes

Virtual Special issue on

St. Petersburg Symposium on TB & Mycobacteria.

Analysis of Mycobacterium tuberculosis genetic lineages circulating in Riga and Riga region, Latvia, isolated between 2008 and 2012.

The running title: Molecular epidemiology of tuberculosis in Latvia.

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Abstract

Although the number of new tuberculosis (TB) cases registered per year has decreased by 3-fold between 2001 and 2017 in Latvia, the TB incidence and rates of multidrug resistant TB in this Baltic country remain substantially higher than in most other European countries. Molecular typing methods of *Mycobacterium tuberculosis* (MTB) play an important role both in clinical studies of the disease and the epidemiological investigations, allowing to describe and characterize the pathogen's population structure and spread of particular genotypes. Aim of this study was to examine the prevalence of MTB lineages in Riga and Riga region of Latvia within a five-year period (2008 – 2012), and to evaluate the discriminatory power (DP) of spoligotyping, standard 24-locus MIRU-VNTR and IS6110-RFLP methods in this setting.

The results showed that the main MTB spoligotype families were Beijing (25.3%) and LAM (24.3%), followed by T (22.1%), Ural (11.2%), Haarlem (6.6%) and X superfamily (3.4%). This distribution remained stable over the five consecutive years. 67.6% of MTB isolates were pan-susceptible, and 32.4% were resistant to any drug; multi-drug resistance was found in 5.8% of MTB strains, and 7.6% of MTB isolates were extensively drug-resistant. Drug resistance was associated with SIT1, SIT283 and SIT42 genotypes, while SIT1 and SIT42 were overrepresented among multi drug-resistant MTB strains. Overall, DP of spoligotyping method alone was 0.8953, while DP of both 24-locus MIRU-VNTR analysis and IS6110 RFLP was higher (DP=0.9846 and 0.9927, respectively), mainly due to the improvement of the resolution for the Beijing strains.

In conclusion, this work represents the first comprehensive molecular epidemiological description of TB in Latvia, highlighting the high genetic diversity of MTB strains circulating in Riga and Riga region. In combination with detailed epidemiological data this approach was

46 helpful for the in-depth understanding of epidemiological processes in settings where the Next-
47 Gen sequencing is not available as a routine method.

48

1. Introduction

The epidemiological situation of tuberculosis (TB) in Latvia, a country in the Baltic region of the northeastern Europe, during the last decade has improved, with 1729 (73.5 per 100 000 population) and 483 (24.8 per 100 000 population) new cases registered in year 2001 and 2017, respectively. However, this incidence remains substantially above the average incidence in most other European countries. Furthermore, Latvia is among the multidrug-resistant tuberculosis (MDR-TB) middle burden countries in the world: there were 36 (7.5%) notified MDR TB cases in 2017 (data from The Centre for Disease Prevention and Control of Latvia).

Molecular typing methods of *Mycobacterium tuberculosis* (MTB) isolates play an important role both in clinical studies of TB and in epidemiological investigations. The description of predominant MTB lineages, diversity of strains and clustering rates is necessary for monitoring the spread of TB in a particular geographic region or epidemiological settings. While several genotyping methods are available to date, all these methods have their own particular advantages and disadvantages in particular settings and they differ with their sensitivity, reproducibility, resolution, and discriminatory power (DP) (Supply et al., 2000; van Soolingen, 2001; Supply et al., 2001; Gori et al., 2005; Supply et al., 2006; reviewed in Merker et al., 2017). Compared to classical typing methods, whole genome sequencing (WGS) method provides the most precise and comprehensive identification and characterization of MTB strain genotypes. However, several barriers for clinical adoption exists including costs and the lack of bioinformatic expertise among clinical microbiologists (Satta et al., 2018). Among the classical typing methods, restriction fragment length polymorphism analysis of IS6110 (IS6110-RFLP), which have been previously known as the first gold standard for MTB strain typing, is time-consuming and needs good quality, purified and concentrated DNA (van Soolingen, 2001). PCR-

based methods such as spoligotyping (Kamerbeek et al., 1997) and 24-locus mycobacterial interspersed repetitive unit variable-number tandem repeat (MIRU-VNTR) typing (Supply et al., 2006) are rapid, easily performed, and can be done with very small quantities of crude DNA extracts. Because of its resolution power, generally superior compared to spoligotyping and in the same range as IS6110 RFLP (Supply et al., 2006; De Beer et al., 2013), molecular clustering based on the latter method has been and is still largely used as a proxy to identify recent transmission of MTB strains, when WGS is not accessible.

Riga is the capital of Latvia and is also the largest city in the Baltic States. With approximately one million inhabitants (year 2017), Riga city and Riga region is home to ~52% of Latvia's population (Central Statistical Bureau of Latvia, csb.gov.lv.). The aim of this study was to examine the prevalence of MTB lineages in Riga and Riga region of Latvia within the five year period (2008 – 2012), and to evaluate the DP of spoligotyping, MIRU-VNTR and IS6110 RFLP methods for genotyping of MTB clinical isolates in this specific setting. This is the first comprehensive molecular epidemiological study of TB in Latvia.

2. Materials and methods

2.1. Study Setting and Design

The Center of Tuberculosis and Lung Diseases, Riga East University Hospital plays a central role in TB management in the country. The Center handles all MTB isolates for the Riga and Riga region, and oversees routine diagnosis, and provides phenotypic drug sensitivity testing.

2.2. Mycobacterial isolates and epidemiological data

The source of the MTB isolates was mainly sputum or induced sputum, and occasionally other biological samples such as urine, faeces, bronchial aspirates, biopsy samples, pleural effusion and operation material, from TB patients living in Riga and Riga region and admitted to Center of Tuberculosis and Lung Diseases, Riga East University Hospital between January 2008 and December 2012. Patient diagnosis was confirmed both microscopically and by culturing on solid Lowenstein-Jensen (LJ) medium for 4–6 weeks. Only one isolate from each patient at time of diagnosis was included in this study.

Epidemiological and clinical data for this study was provided by the National TB registry of Latvia.

2.3. DNA extraction and genotyping

Mycobacterial chromosomal DNA was isolated from mycobacterial colonies grown on Lowenstein Jensen (LJ) media using Cetyltrimethyl Ammonium Bromide (CTAB) method (van Soolingen et al., 1991). Spoligotyping was used to identify MTB genotype for all available clinical isolates. Spoligotyping was performed using commercially available kits (Isogen Life Science, Netherlands; later Ocimum Biosolutions, India) following previously described standard protocol (Kamerbeek et al., 1997). Spoligotyping results were analysed and compared with those in SITVIT2 MTBC database was used for discrimination of MTB spoligotype families (SIT numbers and families) (Couvin D et al., 2018).

24-locus MIRU-VNTR typing was performed using PCR followed by capillary sequencer analysis, as described in (Supply et al., 2006). The 24-allele profiles were analyzed with the tools implemented in the MIRU-VNTRplus online database (Weniger et al., 2010).

DNA fingerprinting with IS6110 as a probe was performed by using standardized protocols as described elsewhere (van Embden et al., 1993). The BioNumerics software Version 5.3 (Applied Maths NV, Sint-Martens-Latern, Belgium) was used for data analysis.

2.4. Drug susceptibility testing

MTB drug susceptibility testing (DST) for the 1st line (isoniazid, rifampicin, ethambutol and pyrazinamide) and 2nd line drugs (streptomycin, ofloxacin, kanamycin, amikacin, capreomycin, ethionamide, cycloserine; para-aminosalicylic acid) was carried out for all strains using the method of absolute concentrations on the solid LJ medium and Bactec MGIT 960 system (Becton Dickinson, Heidelberg, Germany) according to WHO recommendations (WHO, 2018). The laboratory is externally quality assured according to the requirements of standard LVS EN ISO:15189-2013.

2.5. Statistical analysis

The 2-sided Fisher's exact test was employed to determine a *P* value (GraphPad Software, La Jolla, California, USA). A *P* value equal to or less than 0.05 was considered significant. The distribution of the spoligotypes among different age groups was analyzed with principal components analysis (PCA) in R using FactoMineR (Lê et al., 2008). The discriminatory power of each method was calculated by the Hunter-Gaston discriminatory index (HGDI).

3. Results

3.1. Epidemiological data of TB patients

From January 2008 to December 2012, 4241 new TB cases were recorded in Latvia including 1625 TB cases (38.3 %) in Riga and Riga region. Among these cases 7.71% (327/4241) were children (age 0 - 17 years); in Riga and Riga region the proportion of pediatric cases was 9.17% (149/1625). In total, in Riga and Riga region, 1308 cases were culture-positive, and 908 MTB clinical isolates were available for this study.

Table 1 presents an overview of the demographic and clinical characteristics of culture-positive TB patients included in this study. The male/female ratio was 581/327, the median age was 42 (1-89 years old). The majority of patients were 21-60 years old (775 individuals, 85.3%) and were born in Latvia (813 individuals, 89.5%). Within the youngest age group (i.e. individuals 0-20 years old) 16 MTB isolates were from children. Pulmonary TB was diagnosed in 861 (94.8%) cases, while 13 (1.4%) patients had extra-pulmonary TB. Both types of disease were diagnosed in 33 (3.6%) individuals.

3.2. MTB isolate diversity by spoligotyping

Spoligotyping analysis of 908 MTB clinical isolates revealed a total of 138 different patterns (DP: 0.9034) (Supplemental Table S1).

Of 908 isolates, 821 isolates were divided into 51 clusters (cluster size 2-224 isolates). In total, 846 samples (93.2%) showed shared-types or SITs when compared to the SITVIT2 MTBC database. In contrast, only 11 MTB isolates (1.2%) belonged to known orphan spoligotypes, while 51 isolates (5.6% of all samples) corresponded to a total of 38 new spoligotyping patterns that have not yet been reported (Supplemental Table S1). The spoligotyping results showed that

almost equal proportions of MTB strains, Latvia, belonged to the Beijing and LAM lineages in Riga and the Riga region (25.4% and 26.5%, respectively) followed by T lineage (20.3%) (Table 2). Less prevalent genotypes in Riga region were Haarlem (8.5%), Ural (7.9%) and X superfamily (2.4%), while Central Asian and East-African Indian strains were absent. Other spoligotypes accounted for 8.9% of all MTB isolates analysed. The distribution of the MTB spoligotype families within the 5 year period was further analyzed, and the results are shown in Table 2. A slight fluctuation in the distribution of MTB families within different years was observed, however, this difference was not statistically significant ($P>0.05$). Similarly, fluctuations in the distribution of most prevalent MTB spoligotypes (prevalence $\geq 1\%$) within this study period were not statistically significant (Supplemental Table S2).

The Beijing family was represented by four spoligotypes; SIT1 was the dominant spoligotype as it was detected in 224 (24.7%) of all studied clinical isolates. SIT190 was found in only three samples (0.3%), while both SIT255 and SIT265 spoligotypes were detected in 2 samples each (0.2%). By contrast, higher spoligotype diversity was observed for LAM, T, and Haarlem families. The LAM family comprised 241 clinical isolates and was represented by 19 spoligotypes including isolates belonging to LAM-RUS spoligotypes SIT254, 264, 496, 1240 and 2246. Further, 184 MTB isolates that belonged to the T family showed 32 different spoligotypes, while the Haarlem family consisted of 77 studied isolates and was represented by 13 spoligotypes (Supplemental Table S1). Overall, spoligotypes with a prevalence greater than 10% among all isolates included SIT1 (Beijing, 24.7%), SIT254 (LAM-RUS, 11.9%) and SIT53 (T family, 11.0%), while spoligotypes with a prevalence greater than 2% included SIT42, SIT283, SIT766, SIT262, SIT40 and SIT119; other spoligotypes with a prevalence lower than 2% were represented by 280 (30.8%) MTB isolates in total (Table 1).

3.3. Association between spoligotypes and TB patients` characteristics

Possible associations between spoligotypes and TB patients` characteristics were analyzed by PCA analysis followed by statistical evaluation. Upon PCA analysis, age-related differences were interestingly revealed among spoligotype patterns. In a score plot, the spoligotype SIT1 as well as spoligotypes with a prevalence less than 2% in the population (collectively named as others in Fig. 1) were clearly separated; on the other hand, the PCA showed very close distances between SIT254, SIT53, SIT42, SIT283, SIT766, SIT262, SIT40 and SIT119 for the age groups studied (Figure 1). Indeed, the prevalence of SIT1 in the individuals who were older than 60 years of age was decreased, while the prevalence of rare spoligotypes in older individuals was significantly increased in comparison with young (age ≤ 20 years old) patients ($P = 0.0374$) (Table 1).

In contrast, the data analysis revealed no differences of the distribution of spoligotypes among male and female patients. Similarly, no significant differences were observed between Latvia-born and foreign-born individuals, and for the type of the disease (Table 1).

3.4. Association between spoligotypes and TB risk factors

Further, possible association between spoligotypes and TB risk factors was explored. In this study, seven different TB risk factors were analyzed which were included in the National TB registry of Latvia since year 2000: housing status, alcohol abuse, drug abuse, HIV infection, imprisonment, malnutrition and reported contact with a TB patient (Table 3). The majority of patients had a permanent home (92.2%), while 70 (7.7%) patients were homeless. Of the patients for whom HIV status was known (99.7%), 139 (15.3%) individuals were HIV-positive. Alcohol

abuse as a risk factor was detected for 270 (29.7%) patients, while 115 (12.7%) individuals were recorded as being in prison, and 79 (8.7%) - using drugs. Malnutrition was recorded for a half of all patients (51.1%), while less than 30% of individuals have reported the contact with TB patient (27.1%). The presence of any of these seven TB risk factors was detected in the majority of patients (76.5%). Overall, the prevalence of the main spoligotypes among individuals with risk factors was similar to those observed in non-risk patients ($P>0.05$) (Table 3). However, statistically significant difference was detected in the prevalence of both SIT1 and SIT53 spoligotypes depending on the housing status of the patients: SIT53 was more often present in the homeless persons, and SIT1 was more often present in patients with permanent home ($P=0.0083$ and $P=0.0424$, respectively). In addition, the spoligotype SIT262 was more often detected in former prisoners (7.0% vs 1.9%, $P=0.005$).

3.5. Drug resistance analysis

Analysis of drug resistance profiles showed that 67.6% of MTB isolates were pan-susceptible (n=614) and 32.4% were resistant to any drug (n=294) (Table 4). The distribution of spoligotypes among drug resistant isolates was as follows: 43.2% SIT1 (Beijing, n=127), 9.9% SIT42 (LAM family, n=29), 5.8% SIT53 (T family, n=17), 14.6% SIT283 (Haarlem, n=43); all other spoligotypes accounted for 26.5% (n=78) of drug-resistant MTB strains. Spoligotypes with a drug resistance prevalence greater than 30% among the isolates included SIT1 (Beijing, 56.7%), SIT42 (LAM, 56.9%), SIT264 (LAM-RUS, 66.7%), SIT283 (Haarlem, 100%) and SIT1292 (Ural, 41.7%); statistically significant difference was reached for SIT1, SIT42 and SIT283 ($P\leq 0.05$; Table 4).

In total, MDR and extensively-drug-resistance (XDR) was found in 5.8% (53/908) and 7.6% (69/908) of MTB strains, respectively.

The analysis of the distribution of spoligotypes among MDR/XDR isolates showed a significant overrepresentation of both SIT1 and SIT42 isolates: these spoligotypes accounted for 47.2% and 17.0% of MDR MTB, and for 55.1% and 34.0% of XDR strains, respectively (Table 4).

3.6. Analysis of MTB isolates by different genotyping approaches

From 908 MTB isolates, 411 samples were available for additional *IS6110* RFLP and MIRU-VNTR analyses and thus were used for more in-depth genotypic characterization of MTB isolates in Riga and Riga region. In total, these samples represented 25.3% of all MTB-positive cultures isolated in the 2008 – 2012 time period. Among them, 78 spoligotypes were identified. The isolates belonged to the following MTB families: Beijing (n=104; 25.3%), T (n=91; 22.1%), LAM (n=100; 24.3%), Haarlem (n=27; 6.6%), Ural (n=46; 11.2%) and X (n=14; 3.4%); the remaining 29 (7.1%) represented other families and/or unpublished SITs (Table 2). This overall representation of spoligotypes for these 411 isolates was similar to that observed for the full MTB dataset, indicating that this selection was quite representative of the total strain set. Spoligotyping alone identified 78 genotypes, 50 of which were unique; 87.8% of isolates (361 of 411) formed 27 clusters with a cluster range 2-31 samples.

Upon 24-locus MIRU-VNTR typing, 242 different MIRU-VNTR patterns were obtained; 213 MTB isolates (51.8%) were grouped into 44 clusters with 2 to 37 isolates per cluster, while the remaining 198 samples showed a unique MIRU-VNTR profile (Table 5; Supplemental Table 3). Thus, MIRU-VNTR typing had a substantially greater DP (DP=0.9846) in comparison with spoligotyping (DP=0.8953).

248 When 24 MIRU-VNTR-based cluster analysis was performed for the main MTB families
 249 separately, the Beijing family isolates (n=104) were divided into 45 different MIRU-VNTR
 250 genotypes, forming ten clusters and 35 unique genotypes. According to the MLVA MtbC15-9
 251 nomenclature implemented in the MIRU-VNTR Plus database, the 3 most common allelic
 252 profiles were: (1) 94-32 (244233352644425153353823) represented by 37 isolates (35.6%);
 253 (2) 100-32 (244233352644425173353723) represented by 11 isolates (10.6%); and (3) 170-32
 254 (244243352544425153353823) represented by 5 isolates (4.8%) (Table 5; Supplemental Table
 255 3). MTB isolates of the LAM lineage (n=100) were divided into 44 MIRU-VNTR genotypes
 256 forming ten clusters with 2-25 members and 34 unique genotypes. The 3 most common
 257 genetic profiles were following: (1) 121-52 (132244332224125153322622) represented by 25
 258 isolates (25.0%); (2) 843-52 (132254332224125153322622) represented by 15 isolates
 259 (15.0%); (3) 15664-52 (132244332224125153321622) represented by 7 isolates (7.0%).
 260 In contrast, MIRU-VNTR types within the other spoligotype families were relatively more
 261 diverse/less clustered. For instance, MTB isolates of T family (n=91) were divided into 61
 262 MIRU-VNTR genotypes, forming 11 clusters and 50 unique genotypes. The 3 most common
 263 genotypes were represented by 5 (5.5%; 20574-15, 223114322534425153342512), 6 (6.6%;
 264 12677-15, 224243122334225143334322) and 7 isolates (7.7%; 20556-15,
 265 224243122434225143335322), respectively. Likewise, the largest cluster observed among the
 266 isolates part of the Haarlem spoligotype family (n=27) comprised only 4 isolates (14.8%;
 267 3527-15, 223235332434425153324732)), while a second cluster included only 2 isolates
 268 (7.4%). The Ural MTB family members (N=46) formed five clusters, of which 2 were of four
 269 isolates each (163-15, 235237232244425113323632 and 16668-15,
 270 215237232244425113323732); three other clusters had three isolates each. The X family

members (n=14) have formed three clusters which had only two isolates each (Supplemental Table 3).

Next, we applied *IS6110* RFLP typing to all 411 isolates. In total, 257 different patterns were obtained. 207 isolates (50.4%) were grouped into 53 clusters, and the calculated DP for the RFLP genotyping method in our dataset was as high as 0.9927 (Table 5). Importantly, in the case of Beijing strains, *IS6110* RFLP typing showed superior resolution of the genotypes (DP=0.9766) to both spoligotyping and MIRU-VNTR typing (DP=0.0571 and 0.8615, respectively).

When all three genotyping methods were combined, only 119 MTB isolates (29.0%) were grouped into 39 clusters with 2 to 12 isolates per one cluster, while the remaining 292 samples have shown a unique RFLP and/or MIRU-VNTR/spoligotype patterns (Supplemental Table 4). The largest cluster was composed of 12 MTB isolates belonged to SIT254 (representative of LAM-RUS family). In total, the LAM lineage isolates formed 9 clusters with 2-12 isolates, the Beijing genotype isolates - 9 clusters (2-8 isolates), T family – 11 clusters (2-4 isolates), Ural – four clusters (2-3 isolates), and X – three clusters with 2, 3 and 4 isolates each. In addition, two clusters with two isolates each were obtained for the new spoligotypes (spoligotype 757777777720761 and 477777663760771) (Supplemental Table 4).

4. Discussion

Although MTB genotypes were previously studied in association with drug resistance in Latvia (Tracevska et al., 2003; Nodieva et al., 2010; Bauskenieks et al., 2014), this is the first comprehensive report describing the molecular epidemiology of TB in Riga and Riga region,

293 which is home for more than 50% of Latvia's population. The analysis of the prevalence of the
294 main MTB spoligotypes within the five-year time frame revealed the presence of 138
295 spoligotypes. Among them, 38 spoligotypes representing 5.6 percent of the 908 study isolates
296 had not been previously reported to the global SITVIT database. At the other extremity, the 3
297 most common spoligotypes, namely SIT1 (Beijing family), SIT53 (T family) and SIT254
298 (LAM family) accounted altogether for approximately one-half of all MTB isolates, and
299 represented 24.7, 11.0 and 11.9% of isolates, respectively. These patterns are also among the
300 most common SITs in the world (Demay et al., 2012). In addition to be dominant in East Asia,
301 strains with the canonical Beijing SIT1 spoligotype are present in all seven geographic areas
302 of the world (Merker et al, 2015). In Europe, Beijing isolates were previously reported to be
303 very common in the Latvia's eastern and northern neighboring countries, Russia and Estonia,
304 often in association with drug resistance (Toungoussova et al., 2002; Kruuner et al., 2001). In
305 Latvia, too, Beijing genotype was prevalent among MDR-TB patients (Tracevska et al., 2003).
306 More recent data indicated that the XDR *M. tuberculosis* strain population in northwestern
307 Russia is heavily dominated by Beijing genotype isolates (Vyazovaya et al., 2015). In Pskov
308 oblast, which is located in northwestern Russia on the Russian/Latvian border, the Beijing
309 genotype was the most prevalent followed by LAM, T, Haarlem, Ural, and Manu2 families
310 (Mokrousov et al., 2012).

311 In this study, in total, 32.4% of MTB isolates were resistant to any drug, and 13.4% were
312 MDR/XDR strains. Unsurprisingly, Beijing genotype was highly associated with drug
313 resistance, as 43.2% of drug-resistant isolates belonged to the spoligotype SIT1. SIT1 was also
314 the predominant genotype among MDR/XDR isolates: approximately half of all MDR and
315 XDR isolates (i.e. 47.2% and 55.1%, respectively) belonged to this spoligotype. While SIT1

was also the most common spoligotype in our study, the predominance of this genotype among drug-resistant isolates is not related only to the simple majority. The analysis of drug resistance profiles showed that resistance to any drug and MDR/XDR phenotype was observed in 56.7% and 28.1% of the SIT1 strains, respectively. Both proportions were statistically larger when compared to the proportion of drug-resistant and MDR/XDR strains among the total number of MTB isolates (i.e. 32.4% and 13.4%, respectively). MIRU-VNTR analysis of Beijing isolates in Latvia showed the dominance of MTB strains belonged to clusters 94-32 (35.6% of Beijing isolates) and 100-32 (10.6%) which were reported to be the most common cross-border molecular clusters of MDR TB in Europe (ECDC, 2016). Similarly, Beijing clusters 94-32 and 100-32 were the dominant MTB genotypes in Central Asia, and the clonal spread of resistant TB strains, particularly of the Beijing lineage, was highlighted as a root of the MDR-TB epidemic in Central Asia (Engström et al., 2019).

However, the distribution of Beijing strains was not solely responsible for the transmission of drug-resistant TB in Latvia. Less prevalent genotypes among drug-resistant isolates were SIT283 (Haarlem, 14.6%), SIT42 (LAM, 9.9%), SIT53 (T family, 5.8%) and SIT264 (LAM-RUS, 2.7%), followed by other spoligotypes with the prevalence less than 2% each. The proportion of drug-resistant isolates among SIT42, SIT264 and SIT283 samples (56.9%, 66.7% and 100%, respectively), as well as the proportion of MDR/XDR isolates among SIT42 samples (52.9%) was significantly increased. Along with Beijing strains, the combination of spoligotyping/MIRU-VNTR analysis revealed high clustering rate for LAM family in Latvia; the two most common types were 121-52 and 843-52, which accounted for 25.0% and 15.0% of LAM isolates, respectively. While SIT254 was not associated with drug resistance, as 95.4% of isolates were pan-susceptible in our study, the distribution of other

LAM family genotypes, including SIT42 and SIT264, is of high importance. These findings indicate that the high burden of TB drug resistance in Latvia is at least partially related to the circulation of MTB strains of specific genotypes. Thus, genotyping of MTB strains is highly important to identify significant factors involved in the distribution of resistance strains which, in turn, may affect TB control programs.

Overall, the prevalence of different genotype families in Latvia seen in this study resembles those reported for the neighboring Estonia, northwestern Russia and, to a smaller extent, Belarus and Ukraine (where Ural genotypes were not observed according to previous reference reports) (Figure 2) (Chernyaeva et al., 2012; Mokrousov et al., 2012; Toit et al., 2014; Zalutskaya et al., 2013; Nikolaevskaya et al., 2016;). Relatively to these Baltic and Eastern countries, the prevalence of Beijing genotypes is substantially lower in Western Europe and Scandinavia, and the proportion of isolates with spoligotype patterns typical of the Euro-American lineage (alias lineage 4) of MTB is archetypally much higher (Figure 2) (Demay et al., 2012; Smit et al., 2013).

Interestingly, among the 51 isolates with a total of 38 novel spoligotypes that were discovered among circulating MTB strains in Riga region, six shared the same spoligotype 477777663760771, unreported in any other world place thus far. These isolates might thus represent a genotype specific to Latvia. Further studies would help to explore the frequency and underlying clonal/genome diversity of this spoligotype more in details.

Importantly as well, the distribution pattern of the most common genotypes with a dominance of Beijing, LAM and T spoligotypes remained stable over the five-year study period, indicating an overall unchanged prevalence and circulation of different MTB strains in the country and society. Similar result was obtained for the distribution of the most common

spoligotypes including drug resistance-related SIT1, SIT42 and SIT283. This stability at least within this time span limits contrasts with the substantial changes in MTB strain population structures observed over time in other European countries like Ireland and Germany, associated with human migration and mobility (Roycroft et al., 2018; Andrés et al., 2017). These results is consistent with the fact that the vast majority of TB patients in this study, i.e. 89.5%, were local born.

While we did not find any associations of spoligotypes with patients` characteristics such as gender, country of birth or type of the disease, our PCA results showed that the SIT1 spoligotype is more frequent in 21–50 year-old adults while the rare spoligotypes were more frequent in elderly patients. In keeping with the notion that the majority of infected individuals remain asymptomatic over most or all of their lifetime (Zumla et al. 2013), and many of the TB cases in the elderly result from reactivation of a latent MTB infection acquired at much younger ages (Negin et al. 2015), the association of these rare spoligotypes with older patients might reflect genotypes that were more prevalent in the past but have become less abundant nowadays.

We also found that SIT53 (T family, representing a generic spoligotype signature that can be shared by different branches of the Euro-American lineage of *M. tuberculosis*) isolates were more common in homeless patients, while SIT1 spoligotype (Beijing family) was significantly more frequent in patients with permanent home than in homeless persons, and SIT262 (Ural family) was associated with imprisonment. Prisons are settings in which TB transmission occurs and the incidence of TB in prisons is typically several fold higher compared to the general population (reviewed in Biadglegne et al., 2015). Ural family strains have not previously been associated with increased transmissibility (Mokrousov, 2012).

385 However, additional environmental, social, and host related risk factors (like history of
386 previous imprisonment in other (Eastern) countries, of contacts with prisoners with such a
387 history) could facilitate the spread of particular TB strains in these places (Biadglegne et al.,
388 2015). Similarly, low socioeconomic status and enhanced exposure in crowded places such as
389 homeless shelters may have an impact on the transmission dynamics of particular strains (part
390 of the Euro-American lineage in this case) in specific patient groups, such as homeless people.
391 Also, recent report of Sinkov and colleagues highlighted the recent emergency of Ural Clade C
392 (belongs to SIT262) in Eastern European countries Moldova and Belarus which was
393 significantly associated with XDR or pre-XDR status (Sinkov et al., 2019). In our study, the
394 majority of SIT262 isolates were pan-susceptible (22 of 23), while drug resistant strains
395 including those with MDR phenotype were found among isolates belonging to other Ural
396 family spoligotypes SIT35, 1292 and 3344. These findings demonstrated the circulation of
397 Ural strains with concern for drug resistance and the need for detection and surveillance of
398 these strains in Eurasia to prevent further dissemination.

399 Consistently with multiple previous studies, our results show that, although it is a rapid
400 and convenient genotyping method for broad identification of genotype families in clinical
401 strains (Gori et al., 2005) (Millet et al., 2007), spoligotyping cannot be used alone in order to
402 investigate the possible epidemiological links between patients, especially in settings
403 dominated by the Beijing lineage. Isolates with Beijing spoligotypes were the most common in
404 our study, resulting in a spoligotyping clustering rate of 97.1% (101/104 isolates) and a
405 calculated DP for as low as 0.057 for these isolates. Unsurprisingly as well, the 24-locus
406 MIRU-VNTR analysis clearly increased the overall resolution of the obtained MTB genotypes
407 (calculated DP=0.9846), and especially among isolates with a Beijing spoligotype with a

resulting clustering rate of 66.4% (69/104) among them. Likewise, IS6110 RFLP typing had a much higher resolution in comparison with spoligotyping (DP=0.9927), and in the case of Beijing lineage the DP was the highest among the methods applied (DP=0.9766). Given that spoligotyping is both an appropriate routine method for a clinical laboratory in Latvia and a useful screen to identify genotype families, a combination of this method with at least one of the two other molecular genotyping approaches should be used in order to correctly interpret typing results for the needs in epidemiological tracing.

In conclusions, we report the first comprehensive molecular epidemiological study of TB in Latvia. This study highlighted the high genetic diversity of *M. tuberculosis* strains circulating in Riga and Riga region. The combination of the spoligotyping with the 24-locus MIRU-VNTR and IS6110 RFLP clearly increased the overall resolution of the MTB genotypes. In combination with detailed epidemiological data, this approach was helpful for a first in-depth understanding of epidemiological processes in a settings where the Next-Gen sequencing is not available as a routine method.

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Figure captures

Figure 1. Principal component analysis (PCA) of the *M. tuberculosis* spoligotypes in age groups. PCA map showed remarkable segregation of SIT1 and spoligotypes with a prevalence less than 2% in the population (collectively named as Others).

Figure 2. Distribution of *M. tuberculosis* lineages in Latvia and neighboring countries. Data were obtained: Latvia, this study; Russia, Mokrousov et al., 2015; Estonia, Vyazovaya et al., 2018; Finland, Smit et al., 2013; Sweden, Brudey et al., 2004; Belarus, Zalutskaya et al., 2013; Ukraine, Nikolaevskaya et al., 2016; Poland, SITWIT database; Germany, SITVIT database; Denmark, SITVIT database.

Table(s)

Table 1. Characteristics of patients in Riga and Riga region, 2008-2012 (n=908) and association between main spoligotypes.

[illegible]

Table 2. Prevalence of MTB spoligotype families in Riga and Riga region, 2008 – 2012.

Year	Registered NEW TB cases in Riga and Riga region (MTB-positive cultures)	MTB isolates in this study No. (%)*	Spoligotype family, No. of strains (%)						
			Beijing	LAM	T	Haarlem	Ural	X	Others
2008	354 (288)	181 (62.8)	41 (22.7)	50 (27.6)	38 (21.0)	23 (12.7)	9 (5.0)	2 (1.1)	18 (9.9)
2009	305 (253)	212 (83.8)	55 (25.9)	42 (19.8)	41 (19.3)	14 (6.6)	27 (12.7)	9 (4.3)	24 (11.3)
2010	305 (245)	183 (74.7)	40 (21.9)	52 (28.4)	37 (20.2)	14 (7.7)	17 (9.3)	5 (2.7)	18 (9.8)
2011	315 (250)	180 (72.0)	50 (27.8)	54 (30.0)	37 (20.6)	16 (8.9)	9 (5.0)	4 (2.2)	10 (5.6)
2012	346 (272)	152 (56.0)	45 (29.6)	43 (28.3)	31 (20.4)	10 (6.6)	10 (6.6)	2 (1.3)	11 (7.2)
Total	1625 (1308)	908 (69.4)	231 (25.4)	241 (26.5)	184 (20.3)	77 (8.5)	72 (7.9)	22 (2.4)	81 (8.9)
Set of samples **	-	411 (25.3)	104 (25.3)	100 (24.3)	91 (22.1)	27 (6.6)	46 (11.2)	14 (3.4)	29 (7.1)

* The proportion of the MTB-positive cultures.

** Set of MTB samples which were genotyped by all three genotyping methods (Spoligotyping, 24-loci MIRU-VNTR and IS6110 RFLP (n=411)).

Table 3. Spoligotyping of 908 M. tuberculosis clinical samples and association between main spoligotypes and patients` risk factors.

Risk factor		MTB isolates, No. (%)	Spoligotype, No. of strains (%)									
			SIT1 N=224 (24.7)	SIT254 N=108 (11.9)	SIT53 N=100 (11.0)	SIT42 N=51 (5.6)	SIT283 N=43 (4.7)	SIT766 N=35 (3.9)	SIT262 N=23 (2.5)	SIT40 N=23 (2.5)	SIT119 N=21 (2.3)	Others N=280 (30.8)
Housing status	Permanent home	837 (92.2)	214 (25.6)*	100 (12.0)	85 (10.2)**	48 (5.7)	41 (4.9)	31 (3.7)	20 (2.4)	21 (2.5)	17 (2.0)	260 (31.1)
	Homeless	70 (7.7)	10 (14.3)	8 (11.4)	15 (21.4)	3 (4.3)	2 (2.9)	4 (5.7)	3 (4.3)	1 (1.4)	4 (5.7)	20 (28.6)
	Unknown	1 (0.1)										
Alcohol abuse	Yes	270 (29.7)	67 (24.8)	24 (8.9)	35 (13.0)	13 (4.8)	12 (4.4)	11 (4.1)	10 (3.7)	6 (2.2)	8 (3.0)	84 (31.1)
	No	637 (70.2)	157 (24.7)	84 (13.2)	65 (10.2)	38 (6.0)	31 (4.9)	24 (3.8)	13 (2.0)	17 (2.7)	13 (2.0)	195 (30.6)
	Unknown	1 (0.1)										
Drug abuse	Yes	79 (8.7)	18 (22.8)	15 (19.0)	6 (7.6)	3 (3.8)	3 (3.8)	2 (2.5)	4 (5.1)	2 (2.5)	3 (3.8)	23 (29.1)
	No	828 (91.2)	206 (24.9)	93 (11.2)	94 (11.4)	48 (5.8)	40 (4.8)	33 (4.0)	19 (2.3)	21 (2.5)	18 (2.2)	256 (30.9)
	Unknown	1 (0.1)										
HIV positive	Yes	139 (15.3)	38 (27.3)	23 (16.6)	10 (7.2)	8 (5.8)	8 (5.8)	3 (2.2)	5 (3.6)	2 (1.4)	5 (3.6)	37 (26.6)
	No	768 (84.6)	186 (24.2)	85 (11.1)	90 (11.7)	43 (5.6)	35 (4.6)	32 (4.2)	18 (2.3)	21 (2.7)	16 (2.1)	242 (31.5)
	Unknown	1 (0.1)										
Imprisonment	Yes	115 (12.7)	21 (18.3)	15 (13.0)	13 (11.3)	5 (4.4)	6 (5.2)	4 (3.5)	8 (7.0)***	3 (2.6)	3 (2.6)	37 (32.2)
	No	792 (87.2)	203 (25.6)	93 (11.7)	87 (11.0)	46 (5.8)	37 (4.7)	31 (3.9)	15 (1.9)	20 (2.5)	18 (2.3)	242 (30.6)
	Unknown	1 (0.1)										
Malnutrition	Yes	464 (51.1)	116 (25.0)	50 (10.8)	46 (9.9)	25 (5.4)	20 (4.3)	19 (4.1)	14 (3.0)	12 (2.6)	13 (2.8)	149 (32.1)
	No	442 (48.7)	108 (24.4)	58 (13.1)	54 (12.2)	26 (5.9)	23 (5.2)	16 (3.6)	9 (2.0)	11 (2.5)	8 (1.8)	129 (29.2)
	Unknown	2 (0.2)										
Reported contact with a TB patient	Yes	246 (27.1)	67 (27.2)	30 (12.2)	28 (11.4)	15 (6.1)	12 (4.9)	11 (4.5)	3 (1.2)	7 (2.9)	8 (3.3)	65 (26.4)
	No	661 (72.8)	157 (23.8)	78 (11.8)	72 (10.9)	36 (5.5)	31 (4.7)	24 (3.6)	20 (3.0)	16 (2.4)	13 (2.0)	214 (32.4)
	Unknown	1 (0.1)										
Any of 7 risk factors	Yes	695 (76.5)	175 (25.2)	82 (11.8)	76 (10.9)	41 (5.9)	32 (4.6)	30 (4.3)	17 (2.5)	16 (2.3)	20 (2.9)	206 (29.6)
	No	212 (23.4)	49 (23.1)	26 (12.3)	24 (11.3)	10 (4.7)	11 (5.2)	5 (2.4)	6 (2.8)	7 (3.3)	1 (0.5)	73 (34.4)

* This difference was statistically significant: $P=0.0424$; ** $P=0.0083$; *** $P=0.0050$.

Table 4. Drug susceptibility of MTB isolates of the most common spoligotypes (prevalence greater than 1%) in Riga and Riga region, 2008-2012 (n=908).

SIT**	Spoligotype family	Isolates in study, No. (%)	Pan-susceptible, No (%)	Drug-resistant*, No (%)	MDR, No (%)	XDR, No (%)	Pan-susceptible/drug resistant* proportions within SIT, %	MDR/XDR proportions within SIT, %
1	Beijing	224 (24.7)	97 (15.8)	127 (43.2)	25 (47.2)	38 (55.1)	43.3/56.7	11.2/17.0
20	LAM	10 (1.1)	9 (1.5)	1 (0.3)	1 (1.9)	0	90.0/10.0	10/0
35	Ural	12 (1.3)	9 (1.5)	3 (1.0)	2 (3.8)	0	75.0/25.0	16.7/0
40	T	23 (2.5)	22 (3.6)	1 (0.3)	0	0	95.7/4.3	0/0
42	LAM	51 (5.6)	22 (3.6)	29 (9.9)	9 (17.0)	18 (34.0)	43.1/56.9	17.6/35.3
47	Haarlem	9 (1.0)	8 (1.3)	1 (0.3)	0	0	88.9/11.1	0/0
50	Haarlem	11 (1.2)	8 (1.3)	3 (1.0)	0	0	72.7/27.3	0/0
53	T	100 (11.0)	83 (13.5)	17 (5.8)	4 (7.6)	0	83.0/17.0	4/0
119	X	21 (2.3)	20 (3.3)	1 (0.3)	0	0	95.2/4.8	0/0
254	LAM-RUS	108 (11.9)	103 (16.8)	5 (1.7)	0	1 (1.5)	95.4/4.6	0/0.9
262	Ural	23 (2.5)	22 (3.6)	1 (0.3)	0	0	95.7/4.3	0/0
264	LAM-RUS	12 (1.3)	4 (0.7)	8 (2.7)	1 (1.9)	1 (1.5)	33.3/66.7	8.3/8.3
283	Haarlem	43 (4.7)	0	43 (14.6)	3 (5.7)	0	0/100	7.0/0
766	LAM	35 (3.9)	32 (5.2)	3 (1.0)	0	1 (1.5)	91.4/8.6	0/2.9
1292	Ural	12 (1.3)	7 (1.1)	5 (1.7)	0	0	58.3/41.7	0/0
3344	Ural	9 (1.0)	8 (1.3)	1 (0.3)	0	0	88.9/11.1	0/0
Others		205 (22.6)	160 (26.1)	45 (15.3)	8 (15.1)	10 (14.5)	78.0/22.0	3.9/4.9
Total		908	614	294	53	69	67.6/32.4	5.8/7.6

* Including MDR and XDR isolates

Table 5.

Cluster analysis of *M. tuberculosis* isolates by spoligotyping, 24-locus MIRU-VNTR and IS6110 RFLP genotyping methods.

Genotype (No. of strains)*		Genotyping method		
		Spoligotyping	MIRU-VNTR	IS6110 RFLP
Beijing (104)	No. of clusters	1	10	15
	No. of isolates in clusters	101	69	55
	No. of genotypes	4	45	64
	DP	0.0571	0.8615	0.9776
LAM (100)	No. of clusters	4	10	12
	No. of isolates in clusters	94	66	59
	No. of genotypes	10	44	53
	DP	0.6263	0.9099	0.9459
T family (91)	No. of clusters	8	11	14
	No. of isolates in clusters	80	41	45
	No. of genotypes	19	61	60
	DP	0.6232	0.9829	0.9690
Haarlem (27)	No. of clusters	5	2	2
	No. of isolates in clusters	25	6	10
	No. of genotypes	7	23	19
	DP	0.7692	0.9801	0.9174
Ural (46)	No. of clusters	4	5	6
	No. of isolates in clusters	39	17	22
	No. of genotypes	11	34	30
	DP	0.7913	0.9797	0.9681
X family (14)	No. of clusters	1	3	2
	No. of isolates in clusters	13	6	12
	No. of genotypes	2	11	4
	DP	0.1429	0.967	0.6264
Others (29)	No. of clusters	4	3	2
	No. of isolates in clusters	9	8	4
	No. of genotypes	24	24	27
	DP	0.9852	0.9828	0.9951
TOTAL (411 strains)	No. of clusters	27	44	53
	No. of isolates in clusters	361	213	207
	No. of genotypes	77	242	257
	DP	0.8953	0.9846	0.9927

* DP: discrimination power

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Virtual Special issue on

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Analysis of Mycobacterium tuberculosis genetic lineages circulating in Riga and Riga region, Latvia, isolated between 2008 and 2012.

The running title: Molecular epidemiology of tuberculosis in Latvia.

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Abstract

Although the number of new tuberculosis (TB) cases registered per year has decreased by 3-fold between 2001 and 2017 in Latvia, the TB incidence and rates of multidrug-resistant TB in this Baltic country remain substantially higher than in most other European countries. Molecular typing methods of *Mycobacterium tuberculosis* play an important role both in clinical studies of the disease and the epidemiological investigations, allowing to describe and characterize the pathogen's population structure and spread of particular genotypes. The aim of this study was to examine the prevalence of *M. tuberculosis* lineages in Riga and Riga region of Latvia within a five-year period (2008–2012), and to evaluate the discriminatory power of spoligotyping, standard 24-locus MIRU-VNTR and IS6110-RFLP methods for genotyping of *M. tuberculosis* clinical isolates in this setting.

Analysis of spoligotyping results of 908 isolates showed that the main *M. tuberculosis* spoligotype families were Beijing and LAM (25.3% and 24.3%, respectively) followed by T (22.1%), Ural (11.2%), Haarlem (6.6%) and X superfamily (3.4%). This distribution remained similar over the five consecutive years. Analysis of possible associations between spoligotypes and TB patients' characteristics revealed some age-related differences. Moreover, SIT53 (T) and SIT1 (Beijing) spoligotypes were more often detected among homeless patients and patients with permanent home, respectively ($P=0.0083$ and $P=0.0424$, respectively), and SIT262 was more often identified in former prisoners (7.0% vs 1.9%, $P=0.005$).

Spoligotyping, MIRU-VNTR and IS6110-RFLP results were available for a total of 411 *M. tuberculosis* clinical isolates. Overall, the discriminatory power of spoligotyping method alone was 0.8953, but was very low in case among Beijing family isolates, as expected. The discriminatory power of both 24-locus MIRU-VNTR analysis and IS6110-RFLP was higher

(DP=0.9846 and 0.9927, respectively), mainly due to the improvement of the resolution for the Beijing strains.

~~In conclusion, this work represents the first comprehensive molecular epidemiological description of TB in Latvia, highlighting the high genetic diversity of *M. tuberculosis* strains circulating in Riga and Riga region. In combination with detailed epidemiological data this approach was helpful for the in-depth understanding of epidemiological processes in settings where the Next Gen sequencing is not available as a routine method.~~

Although the number of new tuberculosis (TB) cases registered per year has decreased by 3-fold between 2001 and 2017 in Latvia, the TB incidence and rates of multidrug resistant TB in this Baltic country remain substantially higher than in most other European countries. Molecular typing methods of *Mycobacterium tuberculosis* (MTB) play an important role both in clinical studies of the disease and the epidemiological investigations, allowing to describe and characterize the pathogen's population structure and spread of particular genotypes. Aim of this study was to examine the prevalence of MTB lineages in Riga and Riga region of Latvia within a five-year period (2008 – 2012), and to evaluate the discriminatory power (DP) of spoligotyping, standard 24-locus MIRU-VNTR and IS6110-RFLP methods in this setting.

The results showed that the main MTB spoligotype families were Beijing (25.3%) and LAM (24.3%), followed by T (22.1%), Ural (11.2%), Haarlem (6.6%) and X superfamily (3.4%). This distribution remained stable over the five consecutive years. 67.6% of MTB isolates were pan-susceptible, and 32.4% were resistant to any drug; multi-drug resistance was found in 5.8% of MTB strains, and 7.6% of MTB isolates were extensively drug-resistant. Drug resistance was associated with SIT1, SIT283 and SIT42 genotypes, while SIT1 and SIT42 were overrepresented among multi drug-resistant MTB strains. Overall, DP of spoligotyping method alone was 0.8953,

71 while DP of both 24-locus MIRU-VNTR analysis and IS6110 RFLP was higher (DP=0.9846 and
72 0.9927, respectively), mainly due to the improvement of the resolution for the Beijing strains.
73 In conclusion, this work represents the first comprehensive molecular epidemiological
74 description of TB in Latvia, highlighting the high genetic diversity of MTB strains circulating in
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77 Gen sequencing is not available as a routine method.
78

1. Introduction

The epidemiological situation of tuberculosis (TB) in Latvia, a country in the Baltic region of the northeastern Europe, during the last decade has improved, with 1729 (73.5 per 100 000 population) and 483 (24.8 per 100 000 population) new cases registered in year 2001 and 2017, respectively. However, this incidence remains substantially above the average incidence in most other European countries. Furthermore, Latvia is among the multidrug-resistant tuberculosis (MDR-TB) middle burden countries in the world: there were 36 (7.5%) notified MDR TB cases in 2017 (data from The Centre for Disease Prevention and Control of Latvia).

Molecular typing methods of *Mycobacterium tuberculosis* (MTB) isolates play an important role both in clinical studies of TB and in epidemiological investigations. The description of predominant MTB lineages, diversity of strains and clustering rates is necessary for monitoring the spread of TB in a particular geographic region or epidemiological settings. While several genotyping methods are available to date, all these methods have their own particular advantages and disadvantages in particular settings and they differ with their sensitivity, reproducibility, resolution, and discriminatory power (DP) (Supply et al., 2000; van Soolingen, 2001; Supply et al., 2001; Gori et al., 2005; Supply et al., 2006; reviewed in Merker et al., 2017). Compared to classical typing methods, whole genome sequencing (WGS) method provides the most precise and comprehensive identification and characterization of MTB strain genotypes. However, several barriers for clinical adoption exists including costs and the lack of bioinformatic expertise among clinical microbiologists (Satta et al., 2018). Among the classical typing methods, restriction fragment length polymorphism analysis of IS6110 (IS6110-RFLP), which have been previously known as the first gold standard for MTB strain typing, is time-consuming and needs good quality, purified and concentrated DNA (van Soolingen, 2001). PCR-

based methods such as spoligotyping (Kamerbeek et al., 1997) and 24-locus mycobacterial interspersed repetitive unit variable-number tandem repeat (MIRU-VNTR) typing (Supply et al., 2006) are rapid, easily performed, and can be done with very small quantities of crude DNA extracts. Because of its resolution power, generally superior compared to spoligotyping and in the same range as IS6110 RFLP (Supply et al., 2006; De Beer et al., 2013), molecular clustering based on the latter method has been and is still largely used as a proxy to identify recent transmission of MTB strains, when WGS is not accessible.

Riga is the capital of Latvia and is also the largest city in the Baltic States. With approximately one million inhabitants (year 2017), Riga city and Riga region is home to ~52% of Latvia's population (Central Statistical Bureau of Latvia, csb.gov.lv.). The aim of this study was to examine the prevalence of MTB lineages in Riga and Riga region of Latvia within the five year period (2008 – 2012), and to evaluate the DP of spoligotyping, MIRU-VNTR and IS6110 RFLP methods for genotyping of MTB clinical isolates in this specific setting. This is the first comprehensive molecular epidemiological study of TB in Latvia.

2. Materials and methods

2.1. Study Setting and Design

The Center of Tuberculosis and Lung Diseases, Riga East University Hospital plays a central role in TB management in the country. The Center handles all MTB isolates for the Riga and Riga region, and oversees routine diagnosis, and provides phenotypic drug sensitivity testing.

2.2. Mycobacterial isolates and epidemiological data

The source of the MTB isolates was mainly sputum or induced sputum, and occasionally other biological samples such as urine, faeces, bronchial aspirates, biopsy samples, pleural effusion and operation material, from TB patients living in Riga and Riga region and admitted to Center of Tuberculosis and Lung Diseases, Riga East University Hospital between January 2008 and December 2012. Patient diagnosis was confirmed both microscopically and by culturing on solid Lowenstein-Jensen (LJ) medium for 4–6 weeks. Only one isolate from each patient at time of diagnosis was included in this study.

Epidemiological and clinical data for this study was provided by the National TB registry of Latvia.

2.3. DNA extraction and genotyping

Mycobacterial chromosomal DNA was isolated from mycobacterial colonies grown on Lowenstein Jensen (LJ) media using Cetyltrimethyl Ammonium Bromide (CTAB) method (van Soolingen et al., 1991). Spoligotyping was used to identify MTB genotype for all available clinical isolates. Spoligotyping was performed using commercially available kits (Isogen Life Science, Netherlands; later Ocimum Biosolutions, India) following previously described standard protocol (Kamerbeek et al., 1997). Spoligotyping results were analysed and compared with those in SITVIT2 MTBC database was used for discrimination of MTB spoligotype families (SIT numbers and families) (Couvin D et al., 2018).

24-locus MIRU-VNTR typing was performed using PCR followed by capillary sequencer analysis, as described in (Supply et al., 2006). The 24-allele profiles were analyzed with the tools implemented in the MIRU-VNTRplus online database (Weniger et al., 2010).

DNA fingerprinting with IS6110 as a probe was performed by using standardized protocols as described elsewhere (van Embden et al., 1993). The BioNumerics software Version 5.3 (Applied Maths NV, Sint-Martens-Latern, Belgium) was used for data analysis.

2.4. Drug susceptibility testing

MTB drug susceptibility testing (DST) for the 1st line (isoniazid, rifampicin, ethambutol and pyrazinamide) and 2nd line drugs (streptomycin, ofloxacin, kanamycin, amikacin, capreomycin, ethionamide, cycloserine; para-aminosalicylic acid) was carried out for all strains using the method of absolute concentrations on the solid LJ medium and Bactec MGIT 960 system (Becton Dickinson, Heidelberg, Germany) according to WHO recommendations (WHO, 2018). The laboratory is externally quality assured according to the requirements of standard LVS EN ISO:15189-2013.

2.5. Statistical analysis

The 2-sided Fisher's exact test was employed to determine a *P* value (GraphPad Software, La Jolla, California, USA). A *P* value equal to or less than 0.05 was considered significant. The distribution of the spoligotypes among different age groups was analyzed with principal components analysis (PCA) in R using FactoMineR (Lê et al., 2008). The discriminatory power of each method was calculated by the Hunter-Gaston discriminatory index (HGDI).

3. Results

3.1. Epidemiological data of TB patients

From January 2008 to December 2012, 4241 new TB cases were recorded in Latvia including 1625 TB cases (38.3 %) in Riga and Riga region. Among these cases 7.71% (327/4241) were children (age 0 - 17 years); in Riga and Riga region the proportion of pediatric cases was 9.17% (149/1625). In total, in Riga and Riga region, 1308 cases were culture-positive, and 908 MTB clinical isolates were available for this study. ~~Among them, 1308 cases were culture positive, and a total of 908 MTB clinical isolates were available for this study.~~

Table 1 presents an overview of the demographic and clinical characteristics of culture-positive TB patients included in this study. The male/female ratio was 581/327, the median age was 42 (1-89 years old). The majority of patients were 21-60 years old (775 individuals, 85.3%) and were born in Latvia (813 individuals, 89.5%). Within the youngest age group (i.e. individuals 0-20 years old) 16 MTB isolates were from children. Pulmonary TB was diagnosed in 861 (94.8%) cases, while 13 (1.4%) patients had extra-pulmonary TB. Both types of disease were diagnosed in 33 (3.6%) individuals.

3.2. MTB isolate diversity by spoligotyping

Spoligotyping analysis of 908 MTB clinical isolates revealed a total of 138 different patterns (DP: 0.9034) (Supplemental Table S1).

Of 908 isolates, 821 isolates were divided into 51 clusters (cluster size 2-224 isolates). In total, 846 samples (93.2%) showed shared-types or SITs when compared to the SITVIT2 MTBC database. In contrast, only 11 MTB isolates (1.2%) belonged to known orphan spoligotypes,

while 51 isolates (5.6% of all samples) corresponded to a total of 38 new spoligotyping patterns that have not yet been reported (Supplemental Table S1). The spoligotyping results showed that almost equal proportions of MTB strains, Latvia, belonged to the Beijing and LAM lineages in Riga and the Riga region (25.4% and 26.5%, respectively) followed by T lineage (20.3%) (Table 2). Less prevalent genotypes in Riga region were Haarlem (8.5%), Ural (7.9%) and X superfamily (2.4%), while Central Asian and East-African Indian strains were absent. Other spoligotypes accounted for 8.9% of all MTB isolates analysed. The distribution of the MTB spoligotype families within the 5 year period was further analyzed, and the results are shown in Table 2. A slight fluctuation in the distribution of MTB families within different years was observed, however, this difference was not statistically significant ($P>0.05$). Similarly, fluctuations in the distribution of most prevalent MTB spoligotypes (prevalence $\geq 1\%$) within this study period were not statistically significant (Supplemental Table S2).

The Beijing family was represented by four spoligotypes; SIT1 was the dominant spoligotype as it was detected in 224 (24.7%) of all studied clinical isolates. SIT190 was found in only three samples (0.3%), while both SIT255 and SIT265 spoligotypes were detected in 2 samples each (0.2%). By contrast, higher spoligotype diversity was observed for LAM, T, and Haarlem families. The LAM family comprised 241 clinical isolates and was represented by 19 spoligotypes including isolates belonging to LAM-RUS spoligotypes SIT254, 264, 496, 1240 and 2246. Further, 184 MTB isolates that belonged to the T family showed 32 different spoligotypes, while the Haarlem family consisted of 77 studied isolates and was represented by 13 spoligotypes (Supplemental Table S1). Overall, spoligotypes with a prevalence greater than 10% among all isolates included SIT1 (Beijing, 24.7%), SIT254 (LAM-RUS, 11.9%) and SIT53 (T family, 11.0%), while spoligotypes with a prevalence greater than 2% included

SIT42, SIT283, SIT766, SIT262, SIT40 and SIT119; other spoligotypes with a prevalence lower than 2% were represented by 280 (30.8%) MTB isolates in total (Table 1).

3.3. Association between spoligotypes and TB patients` characteristics

Possible associations between spoligotypes and TB patients` characteristics were analyzed by PCA analysis followed by statistical evaluation. Upon PCA analysis, age-related differences were interestingly revealed among spoligotype patterns. In a score plot, the spoligotype SIT1 as well as spoligotypes with a prevalence less than 2% in the population (collectively named as others in Fig. 1) were clearly separated; on the other hand, the PCA showed very close distances between SIT254, SIT53, SIT42, SIT283, SIT766, SIT262, SIT40 and SIT119 for the age groups studied (Figure 1). Indeed, the prevalence of SIT1 in the individuals who were older than 60 years of age was decreased, while the prevalence of rare spoligotypes in older individuals was significantly increased in comparison with young (age ≤ 20 years old) patients ($P = 0.0374$) (Table 1).

In contrast, the data analysis revealed no differences of the distribution of spoligotypes among male and female patients. Similarly, no significant differences were observed between Latvia-born and foreign-born individuals, and for the type of the disease (Table 1).

3.4. Association between spoligotypes and TB risk factors

Further, possible association between spoligotypes and TB risk factors was explored. In this study, seven different TB risk factors were analyzed which were included in the National TB registry of Latvia since year 2000: housing status, alcohol abuse, drug abuse, HIV infection, imprisonment, malnutrition and reported contact with a TB patient (Table 3). The majority of

patients had a permanent home (92.2%), while 70 (7.7%) patients were homeless. Of the patients for whom HIV status was known (99.7%), 139 (15.3%) individuals were HIV-positive. Alcohol abuse as a risk factor was detected for 270 (29.7%) patients, while 115 (12.7%) individuals were recorded as being in prison, and 79 (8.7%) - using drugs. Malnutrition was recorded for a half of all patients (51.1%), while less than 30% of individuals have reported the contact with TB patient (27.1%). The presence of any of these seven TB risk factors was detected in the majority of patients (76.5%). Overall, the prevalence of the main spoligotypes among individuals with risk factors was similar to those observed in non-risk patients ($P>0.05$) (Table 3). However, statistically significant difference was detected in the prevalence of both SIT1 and SIT53 spoligotypes depending on the housing status of the patients: SIT53 was more often present in the homeless persons, and SIT1 was more often present in patients with permanent home ($P=0.0083$ and $P=0.0424$, respectively). In addition, the spoligotype SIT262 was more often detected in former prisoners (7.0% vs 1.9%, $P=0.005$).

3.5. Drug resistance analysis

Analysis of drug resistance profiles showed that 67.6% of MTB isolates were pan-susceptible (n=614) and 32.4% were resistant to any drug (n=294) (Table 4). The distribution of spoligotypes among drug resistant isolates was as follows: 43.2% SIT1 (Beijing, n=127), 9.9% SIT42 (LAM family, n=29), 5.8% SIT53 (T family, n=17), 14.6% SIT283 (Haarlem, n=43); all other spoligotypes accounted for 26.5% (n=78) of drug-resistant MTB strains. Spoligotypes with a drug resistance prevalence greater than 30% among the isolates included SIT1 (Beijing, 56.7%), SIT42 (LAM, 56.9%), SIT264 (LAM-RUS, 66.7%), SIT283 (Haarlem, 100%) and

SIT1292 (Ural, 41.7%); statistically significant difference was reached for SIT1, SIT42 and SIT283 ($P \leq 0.05$; Table 4).

In total, MDR and extensively-drug-resistance (XDR) was found in 5.8% (53/908) and 7.6% (69/908) of MTB strains, respectively.

The analysis of the distribution of spoligotypes among MDR/XDR isolates showed a significant overrepresentation of both SIT1 and SIT42 isolates: these spoligotypes accounted for 47.2% and 17.0% of MDR MTB, and for 55.1% and 34.0% of XDR strains, respectively (Table 4).

3.65. Analysis of MTB isolates by different genotyping approaches

From 908 MTB isolates, 411 samples were available for additional IS6110 RFLP and MIRU-VNTR analyses and thus were used for more in-depth genotypic characterization of MTB isolates in Riga and Riga region. In total, these samples represented 25.3% of all MTB-positive cultures isolated in the 2008 – 2012 time period. Among them, 78 spoligotypes were identified. The isolates belonged to the following MTB families: Beijing (n=104; 25.3%), T (n=91; 22.1%), LAM (n=100; 24.3%), Haarlem (n=27; 6.6%), Ural (n=46; 11.2%) and X (n=14; 3.4%); the remaining 29 (7.1%) represented other families and/or unpublished SITs (Table 2). This overall representation of spoligotypes for these 411 isolates was similar to that observed for the full MTB dataset, indicating that this selection was quite representative of the total strain set. Spoligotyping alone identified 78 genotypes, 50 of which were unique; 87.8% of isolates (361 of 411) formed 27 clusters with a cluster range 2-31 samples. Upon 24-locus MIRU-VNTR typing, 242 different MIRU-VNTR patterns were obtained; 213 MTB isolates (51.8%) were grouped into 44 clusters with 2 to 37 isolates per cluster, while the remaining 198 samples showed a unique MIRU-VNTR profile (Table 54; Supplemental Table

278 | [32](#)). Thus, MIRU-VNTR typing had a substantially greater DP (DP=0.9846) in comparison
279 | with spoligotyping (DP=0.8953).

280 | When 24 MIRU-VNTR-based cluster analysis was performed for the main MTB families
281 | separately, the Beijing family isolates (n=104) were divided into 45 different MIRU-VNTR
282 | genotypes, forming ten clusters and 35 unique genotypes. According to the MLVA MtbC15-9
283 | nomenclature implemented in the MIRU-VNTR Plus database, the 3 most common allelic
284 | profiles were: (1) 94-32 (244233352644425153353823) represented by 37 isolates (35.6%);
285 | (2) 100-32 (244233352644425173353723) represented by 11 isolates (10.6%); and (3) 170-32
286 | (244243352544425153353823) represented by 5 isolates (4.8%) (Table [54](#); Supplemental
287 | Table [32](#)). MTB isolates of the LAM lineage (n=100) were divided into 44 MIRU-VNTR
288 | genotypes forming ten clusters with 2-25 members and 34 unique genotypes. The 3 most
289 | common genetic profiles were following: (1) 121-52 (132244332224125153322622)
290 | represented by 25 isolates (24.0%); (2) 843-52 (132254332224125153322622) represented by
291 | 15 isolates (15.0%); (3) 15664-52 (132244332224125153321622) represented by 7 isolates
292 | (7.0%).

293 | In contrast, MIRU-VNTR types within the other spoligotype families were relatively more
294 | diverse/less clustered. For instance, MTB isolates of T family (n=91) were divided into 61
295 | MIRU-VNTR genotypes, forming 11 clusters and 50 unique genotypes. The 3 most common
296 | genotypes were represented by 5 (5.5%; 20574-15, 223114322534425153342512), 6 (6.6%;
297 | 12677-15, 224243122334225143334322) and 7 isolates (7.7%; 20556-15,
298 | 224243122434225143335322), respectively. Likewise, the largest cluster observed among the
299 | isolates part of the Haarlem spoligotype family (n=27) comprised only 4 isolates (14.8%;
300 | 3527-15, 223235332434425153324732)), while a second cluster included only 2 isolates

(7.4%). The Ural MTB family members (N=46) formed five clusters, of which 2 were of four isolates each (163-15, 235237232244425113323632 and 16668-15, 215237232244425113323732); three other clusters had three isolates each. The X family members (n=14) have formed three clusters which had only two isolates each (Supplemental Table 32).

Next, we applied *IS6110* RFLP typing to all 411 isolates. In total, 257 different patterns were obtained. 207 isolates (50.4%) were grouped into 53 clusters, and the calculated DP for the RFLP genotyping method in our dataset was as high as 0.9927 (Table 54). Importantly, in the case of Beijing strains, *IS6110* RFLP typing showed superior resolution of the genotypes (DP=0.9766) to both spoligotyping and MIRU-VNTR typing (DP=0.0571 and 0.8615, respectively).

When all three genotyping methods were combined, only 119 MTB isolates (29.0%) were grouped into 39 clusters with 2 to 12 isolates per one cluster, while the remaining 292 samples have shown a unique RFLP and/or MIRU-VNTR/spoligotype patterns (Supplemental Table 43). The largest cluster was composed of 12 MTB isolates belonged to SIT254 (representative of LAM-RUS family). In total, the LAM lineage isolates formed 9 clusters with 2-12 isolates, the Beijing genotype isolates - 9 clusters (2-8 isolates), T family – 11 clusters (2-4 isolates), Ural – four clusters (2-3 isolates), and X – three clusters with 2, 3 and 4 isolates each. In addition, two clusters with two isolates each were obtained for the new spoligotypes (spoligotype 757777777720761 and 477777663760771) (Supplemental Table 43).

4. Discussion

Although MTB genotypes were previously studied in association with drug resistance in Latvia (Tracevska et al., 2003; Nodieva et al., 2010; Bauskenieks et al., 2014), this is the first comprehensive report describing the molecular epidemiology of TB in Riga and Riga region, which is home for more than 50% of Latvia's population. The analysis of the prevalence of the main MTB spoligotypes within the five-year time frame revealed the presence of 138 spoligotypes. Among them, 38 spoligotypes representing 5.6 percent of the 908 study isolates had not been previously reported to the global SITVIT database. At the other extremity, the 3 most common spoligotypes, namely SIT1 (Beijing family), SIT53 (T family) and SIT254 (LAM family) accounted altogether for approximately one-half of all MTB isolates, and represented 24.7, 11.0 and 11.9% of isolates, respectively. These patterns are also among the most common SITs in the world (Demay et al., 2012). In addition to be dominant in East Asia, strains with the canonical Beijing SIT1 spoligotype are present in all seven geographic areas of the world (Merker et al, 2015). In Europe, Beijing isolates were previously reported to be very common in the Latvia's eastern and northern neighboring countries, Russia and Estonia, often in association with drug resistance (Toungoussova et al., 2002; Kruuner et al., 2001). In Latvia, too, Beijing genotype was prevalent among MDR-TB patients (Tracevska et al., 2003). More recent data indicated that the XDR *M. tuberculosis* strain population in northwestern Russia is heavily dominated by Beijing genotype isolates (Vyazovaya et al., 2015). In Pskov oblast, which is located in northwestern Russia on the Russian/Latvian border, the Beijing genotype was the most prevalent followed by LAM, T, Haarlem, Ural, and Manu2 families (Mokrousov et al., 2012).

In this study, in total, 32.4% of MTB isolates were resistant to any drug, and 13.4% were MDR/XDR strains. Unsurprisingly, Beijing genotype was highly associated with drug

resistance, as 43.2% of drug-resistant isolates belonged to the spoligotype SIT1. SIT1 was also the predominant genotype among MDR/XDR isolates: approximately half of all MDR and XDR isolates (i.e. 47.2% and 55.1%, respectively) belonged to this spoligotype. While SIT1 was also the most common spoligotype in our study, the predominance of this genotype among drug-resistant isolates is not related only to the simple majority. The analysis of drug resistance profiles showed that resistance to any drug and MDR/XDR phenotype was observed in 56.7% and 28.1% of the SIT1 strains, respectively. Both proportions were statistically larger when compared to the proportion of drug-resistant and MDR/XDR strains among the total number of MTB isolates (i.e. 32.4% and 13.4%, respectively). MIRU-VNTR analysis of Beijing isolates in Latvia showed the dominance of MTB strains belonged to clusters 94-32 (35.6% of Beijing isolates) and 100-32 (10.6%) which were reported to be the most common cross-border molecular clusters of MDR TB in Europe (ECDC, 2016). Similarly, Beijing clusters 94–32 and 100–32 were the dominant MTB genotypes in Central Asia, and the clonal spread of resistant TB strains, particularly of the Beijing lineage, was highlighted as a root of the MDR-TB epidemic in Central Asia (Engström et al., 2019). However, the distribution of Beijing strains was not solely responsible for the transmission of drug-resistant TB in Latvia. Less prevalent genotypes among drug-resistant isolates were SIT283 (Haarlem, 14.6%), SIT42 (LAM, 9.9%), SIT53 (T family, 5.8%) and SIT264 (LAM-RUS, 2.7%), followed by other spoligotypes with the prevalence less than 2% each. The proportion of drug-resistant isolates among SIT42, SIT264 and SIT283 samples (56.9%, 66.7% and 100%, respectively), as well as the proportion of MDR/XDR isolates among SIT42 samples (52.9%) was significantly increased. Along with Beijing strains, the combination of spoligotyping/MIRU-VNTR analysis revealed high clustering rate for LAM family in Latvia;

the two most common types were 121-52 and 843-52, which accounted for 25.0% and 15.0% of LAM isolates, respectively. While SIT254 was not associated with drug resistance, as 95.4% of isolates were pan-susceptible in our study, the distribution of other LAM family genotypes, including SIT42 and SIT264, is of high importance. These findings indicate that the high burden of TB drug resistance in Latvia is at least partially related to the circulation of MTB strains of specific genotypes. Thus, genotyping of MTB strains is highly important to identify significant factors involved in the distribution of resistance strains which, in turn, may affect TB control programs.

Overall, the prevalence of different genotype families in Latvia seen in this study resembles those reported for the neighboring Estonia, northwestern Russia and, to a smaller extent, Belarus and Ukraine (where Ural genotypes were not observed according to previous reference reports) (Figure 2) (Chernyaeva et al., 2012; Mokrousov et al., 2012; Toit et al., 2014; Zalutskaya et al., 2013; Nikolaevskaya et al., 2016;). Relatively to these Baltic and Eastern countries, the prevalence of Beijing genotypes is substantially lower in Western Europe and Scandinavia, and the proportion of isolates with spoligotype patterns typical of the Euro-American lineage (alias lineage 4) of *M. tuberculosis* is archetypally much higher (Figure 2) (Demay et al., 2012; Smit et al., 2013).

Interestingly, among the 51 isolates with a total of 38 novel spoligotypes that were discovered among circulating MTB strains in Riga region, six shared the same spoligotype 477777663760771, unreported in any other world place thus far. These isolates might thus represent a genotype specific to Latvia. Further studies would help to explore the frequency and underlying clonal/genome diversity of this spoligotype more in details.

Importantly as well, the distribution pattern of the most common genotypes with a dominance of Beijing, LAM and T spoligotypes remained stable over the five-year study period, indicating an overall unchanged prevalence and circulation of different MTB strains in the country and society. Similar result was obtained for the distribution of the most common spoligotypes including drug resistance-related SIT1, SIT42 and SIT283. This stability at least within this time span limits contrasts with the substantial changes in MTB strain population structures observed over time in other European countries like Ireland and Germany, associated with human migration and mobility (Roycroft et al., 2018; Andrés et al., 2017). These results is consistent with the fact that the vast majority of TB patients in this study, i.e. 89.5%, were local born.

While we did not find any associations of spoligotypes with patients` characteristics such as gender, country of birth or type of the disease, our PCA results showed that the SIT1 spoligotype is more frequent in 21–50 year-old adults while the rare spoligotypes were more frequent in elderly patients. In keeping with the notion that the majority of infected individuals remain asymptomatic over most or all of their lifetime (Zumla et al. 2013), and many of the TB cases in the elderly result from reactivation of a latent MTB infection acquired at much younger ages (Negin et al. 2015), the association of these rare spoligotypes with older patients might reflect genotypes that were more prevalent in the past but have become less abundant nowadays.

We also found that SIT53 (T family, representing a generic spoligotype signature that can be shared by different branches of the Euro-American lineage of *M. tuberculosis*) isolates were more common in homeless patients, while SIT1 spoligotype (Beijing family) was significantly more frequent in patients with permanent home than in homeless persons, and

SIT262 (Ural family) was associated with imprisonment. Prisons are settings in which TB transmission occurs and the incidence of TB in prisons is typically several fold higher compared to the general population (reviewed in Biadlegne et al., 2015). Ural family strains have not previously been associated with increased transmissibility (Mokrousov, 2012). However, additional environmental, social, and host related risk factors (like history of previous imprisonment in other (Eastern) countries, of contacts with prisoners with such a history) could facilitate the spread of particular TB strains in these places (Biadlegne et al., 2015). Similarly, low socioeconomic status and enhanced exposure in crowded places such as homeless shelters may have an impact on the transmission dynamics of particular strains (part of the Euro-American lineage in this case) in specific patient groups, such as homeless people.

Also, recent report of Sinkov and colleagues highlighted the recent emergency of Ural Clade C (belongs to SIT262) in Eastern European countries Moldova and Belarus which was significantly associated with XDR or pre-XDR status (Sinkov et al., 2019). In our study, the majority of SIT262 isolates were pan-susceptible (22 of 23), while drug resistant strains including those with MDR phenotype were found among isolates belonging to other Ural family spoligotypes SIT35, 1292 and 3344. These findings demonstrated the circulation of Ural strains with concern for drug resistance and the need for detection and surveillance of these strains in Eurasia to prevent further dissemination.

Consistently with multiple previous studies, our results show that, although it is a rapid and convenient genotyping method for broad identification of genotype families in clinical strains (Gori et al., 2005) (Millet et al., 2007), spoligotyping cannot be used alone in order to investigate the possible epidemiological links between patients, especially in settings dominated by the Beijing lineage. Isolates with Beijing spoligotypes were the most common in

our study, resulting in a spoligotyping clustering rate of 97.1% (101/104 isolates) and a calculated DP for as low as 0.057 for these isolates. Unsurprisingly as well, the 24-locus MIRU-VNTR analysis clearly increased the overall resolution of the obtained MTB genotypes (calculated DP=0.9846), and especially among isolates with a Beijing spoligotype with a resulting clustering rate of 66.4% (69/104) among them. Likewise, IS6110 RFLP typing had a much higher resolution in comparison with spoligotyping (DP=0.9927), and in the case of Beijing lineage the DP was the highest among the methods applied (DP=0.9766). Given that spoligotyping is both an appropriate routine method for a clinical laboratory in Latvia and a useful screen to identify genotype families, a combination of this method with at least one of the two other molecular genotyping approaches should be used in order to correctly interpret typing results for the needs in epidemiological tracing.

In conclusions, we report the first comprehensive molecular epidemiological study of TB in Latvia. This study highlighted the high genetic diversity of *M. tuberculosis* strains circulating in Riga and Riga region. The combination of the spoligotyping with the 24-locus MIRU-VNTR and IS6110 RFLP clearly increased the overall resolution of the MTB genotypes. In combination with detailed epidemiological data, this approach was helpful for a first in-depth understanding of epidemiological processes in a settings where the Next-Gen sequencing is not available as a routine method.

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Figure captures

Figure 1. Principal component analysis (PCA) of the *M. tuberculosis* spoligotypes in age groups. PCA map showed remarkable segregation of SIT1 and spoligotypes with a prevalence less than 2% in the population (collectively named as Others).

Figure 2. Distribution of *M. tuberculosis* lineages in Latvia and neighboring countries. Data were obtained: Latvia, this study; Russia, Mokrousov et al., 2015; Estonia, Vyazovaya et al., 2018; Finland, Smit et al., 2013; Sweden, Brudey et al., 2004; Belarus, Zalutskaya et al., 2013; Ukraine, Nikolaevskaya et al., 2016; Poland, SITWIT database; Germany, SITVIT database; Denmark, SITVIT database.

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