СЕВЕРО-ЗАПАДНОЕ ОТДЕЛЕНИЕ МЕДИЦИНСКИХ НАУК
САНКТ-ПЕТЕРБУРГСКИЙ НАУЧНО-ИССЛЕДОВАТЕЛЬСКИЙ ИНСТИТУТ ЭПИДЕМИОЛОГИИ И МИКРОБИОЛОГИИ ИМЕНИ ПАСТЕРА
САНКТ-ПЕТЕРБУРГСКОЕ РЕГИОНАЛЬНОЕ ОТДЕЛЕНИЕ РОССИЙСКОЙ АССОЦИАЦИИ АЛЛЕРГОЛОГОВ И КЛИНИЧЕСКИХ ИММУНОЛОГОВ (СПб РО РААКИ)

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6. TUBERCULOSIS AND MYCOBACTERIA: MOLECULAR APPROACH*

6.1

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PERFORMANCE OF GENEXPERT MTB/RIF IN THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN MOROCCO

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Tuberculosis is commonly associated with lung diseases, but can also affect other parts of the body (extrapulmonary tuberculosis [EPT]). A rapid diagnosis is essential to initiate a specific and effective treatment. The diagnosis of EPT is a real challenge because of the paucibacillary nature of samples. GeneXpert MTB/RIF is a rapid automated diagnostic test that allows the detection of the presence of *M. tuberculosis* as well as mutations in the hot-spot region of the *rpoB* gene associated with rifampicine resistance. The objective of this study was to evaluate the performance of the GeneXpert MTB/RIF test for the diagnosis of EPT.

We analyzed 304 clinical samples collected in the Laboratory of Mycobacteria and Tuberculosis of Pasteur Institute of Morocco, between 2016 and 2017. Of these samples, 113 were pleural fluids decontaminated using the Petroff method and 191 biopsies (78 lymph nodes and 113 pleural biopsy), decontaminated using the Loeweinstein method. All samples underwent smear microscopy, culture on Loeweinstein–Jensen medium and tested with Xpert MTB/RIF.

The study population included 192 patients, 54.2% were men and 45.8% women. The age of the patients ranged from 2–78 years with the majority of the patients in the age group 25–45 years. The sensitivity of GeneXpert was 51.47% for all samples and 83.3% for lymph nodes.

Our study clearly shows that GeneXpert MTB/RIF test presents limitations in the diagnosis of EPT. In view of these results, it would not be appropriate to use only this technique for the diagnosing of EPT.

6.2

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ANALYSIS OF GENE MUTATIONS ASSOCIATED WITH *MDR AMONG MYCOBACTERIUM T*UBERCULOSIS STRAINS ISOLATED IN MOSCOW REGION

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The aim of this study was to determine the prevalence and variants of mutations in *M. tuberculosis* genes associated with the development of multidrug-resistance (MDR) as well as their correlation with genotypes in the study of clinical isolates obtained from patients with tuberculosis (TB) in hospitals from the Moscow region.

179 randomly selected *M. tuberculosis* clinical isolates from TB patients collected from 2008 to 2016 years were included in this study. One isolate from each patient was used. The molecular characteristics of *rpoB*, *katG* genes and *inhA* promoter, resulting in rifampin and isoniazid resistance (MDR), were obtained by Sanger sequencing. All specimens were subjected to spoligotyping; spoligotypes were compared to SITVIT_WEB database. Pearson χ^2 test was used to check pairwise differences.

All clinical isolates were divided into 2 groups of genotypes according to the results of spoligotyping: Beijing (72.6%, n = 130) and other genotypes collectively named "non-Beijing" genotypes (27.4%, n = 49). Beijing genotype had *rpoB* Ser 531> Leu mutation in 62.9% of cases whereas non-Beijing genotypes in only 15.8% of cases. Other variants of *rpoB* mutations were detected in only 6.3% of Beijing strains versus 28.1% of non-Beijing strains. The wild-type *rpoB* gene was observed in Beijing genotype in 30.8% of cases whereas in non-Beijing genotype in 56.1%. Statistically significant differences were obtained for all comparisons between two groups ($\chi^2 = 9.21$, $p < 0.01$).

We also obtained statistically significant differences in the analysis of combinations of *katG* gene and *inhA* promoter for Beijing and non-Beijing genotypes, respectively: in the case of simultaneous presence of mutations in them, in 13.9% and in 34.9% of cases ($\chi^2 = 8.59$, $P = 0.0036$) or wild-type in 20.4 and 39.5% of cases ($\chi^2 = 20.64$, $p < 0.0001$), as well as in the presence of genetic changes in only *katG* gene, 62.0 and 20.9% ($\chi^2 = 20.64$, $p < 0.0001$). However, no statistically significant differences were noted when comparing *inhA* promoter mutations occurred alone without *katG* mutations which was observed in a small proportions in both genotypes — 3.7 and 4.7%.

We established some specific features in clinical isolates of *M. tuberculosis* in Moscow region.

6.3

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COULD THE NEW INSIGHTS INTO PZA RESISTANCE PROVIDE ROUTE TO SHORTER MORE EFFECTIVE TB THERAPY?

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Pyrazinamide (PZA) is a pro-drug that is transformed into pyrazinoic acid (POA) by mycobacterial PncA enzyme. A very wide range of mutations in *pncA* result in clinical and *in vitro* resistance. In the last two years multiple groups have demonstrated that a low pH is not required for the activity of POA against tuberculosis as was previously widely assumed. Furthermore, laboratory mutants against POA have been generated in multiple laboratories under different conditions. Mutations in a range of genes have been observed but always including *clpC1* and/or *panD*. A direct activity of POA against mycobacterial PanD has been demonstrated but evidence of activity against other genes associated with *in vitro* resistance is disputed or lacking. It has been suggested that PZA is a dirty drug with multiple targets but we recently

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proposed an alternative explanation: POA is primarily active against PanD but PanD is only essential if the bacteria are expressing a stringent response, the other genes associated with resistance in some way disrupt the stringent response and eliminate the sensitive phenotype. This suggests a critical role for the stringent response in the life cycle of *M. tuberculosis* as compounds are being developed that target this pathway we suggest these compounds are particularly promising compounds for the treatment of tuberculosis.

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IDENTIFICATION OF MUTATIONS OF RESISTANCE TO FLUOROQUINOLONES, AMINOGLYCOSIDES AND ETHAMBUTOL IN RIFAMPICIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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The aim of the study was to identify resistance mutations to second-line anti-tuberculosis drugs in patients with *Mycobacterium tuberculosis* clinical samples resistant to rifampicin. Samples of biomaterial from 35 adult residents of the Tyumen region in West Siberia with established by GeneXpert system presence of rifampicin-resistant mycobacteria tuberculosis (MBT) were examined in our study using the MTBDRsl kit (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. The mutations were identified in genes encoding DNA gyrase *(gryA)*, 16S RNA *(rrs)*, and arabinosyltrasferase *(embB)* associated with resistance to fluoroquinolones (FLQ), injectable aminoglycosides/cyclic peptides (AG/CH) and ethambutol (EMB) respectively.

In 9 of the examined samples (25.7%) the MBT resistance to all three groups of drugs was revealed. In the remaining 26 samples, the MBT sensitivity to one or two groups of drugs can be assumed. Samples from 24 patients (92%) were genetically susceptible to AG/CH (in 2/3 cases solo, in 1/3 — in combination with sensitivity to ethambutol (5 samples) and fluoroquinolones (1)). Most samples demonstrated genetic resistance to fluoroquinolones (97%) and ethambutol (80%), and 30% of samples are resistant to aminoglycosides/cyclic peptides.

Among the *gyrA* mutations, 11 were in codon 90 (A90V), 43 — in codon 94 (of which 4 — D94A, 6 — D94N, D94Y, 30 — D94G and 3 — D94H). No mutation in codon 91 (S91P) was detected. In 22 samples 1 mutation was detected, in 4 — 2 mutations, in 6 — 3 mutations, and in 1 — 5 mutations. Among the mutations found in the *rrs* gene, 8 are in codons 1401–1402 (A1401G, C1402T) and 10 — in codon 1484 (G1484T), all mutations in codons 1401–1402 are combined with the presence of a mutation in codon 1484. Among mutations in *embB* (codon 306) in three cases replacement of M306I = 306 ATG/ATA, M306V, in 26 — replacement of M306I = 306 ATG/ATC/ATT was revealed. In 24 cases only one variant of the mutation is found, in 2 — both, and in 18 samples in the presence of a mutation there is no marker of wild type.

In conclusion, preliminary data on the genetic structure of MBT strains resistant to rifampicin and second-line anti-tuberculosis drugs were obtained in tuberculosis patients from the Tyumen Region in Siberia.

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MOLECULAR TYPING OF *MYCOBACTERIUM KANSASII* — A GLOBAL PERSPECTIVE

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To date, over 180 nontuberculous mycobacteria (NTM) species have been identified and almost 30 of these species have been reported as the causative agents of pulmonary and extrapulmonary diseases. *Mycobacterium kansasii* is the sixth most frequently isolated NTM species across the world. The isolation rate of this pathogen, among other NTM, has been calculated at 5% in Europe and 4% globally. In Poland and Slovakia, the recovery of *M. kansasii* from respiratory samples is particularly high, being 36% and 35%, respectively.

The genetic heterogeneity of *M. kansasii* is defined by the presence of seven molecular subtypes. Most of the disease-related strains belong to subtype I and II, while the others (III-VII) have usually been linked to environmental sources. Therefore, subtyping of *M. kansasii* isolates from human samples may be helpful for clinical diagnosis.

The aim of this study was to determine the distribution of *M. kansasii* subtypes among clinical isolates from 19 countries on 4 continents.

A total of 475 isolates recovered between 2000 and 2017 from as many patients with suspected *M. kansasii* disease were analyzed. The isolates were collected from 19 coun-

tries across 4 continents. For PCR restriction-enzyme analysis (PCR-REA) subtyping, protocols described by Telenti et al. (2003) (*hsp65* gene) and Bakuła et. al. (2016) (*tuf* gene) were used. The patients were categorized as having *M. kansasii* disease following the American Thoracic Society 2007 diagnostic criteria.

The vast majority of isolates (392; 82.5%) presented patterns characteristic for subtype I. Forty-three (9%) isolates exhibited subtype II pattern. There were 19 (4%), 2 (0.4%), 2 (0.4%) and 13 (2.7%) isolates representing subtypes III, IV, V, and VI, respectively. Four (0.8%) isolates gave inconsistent results (mixed subtype — I/II). The subtype I–VI isolates were obtained from both disease-associated and non-disease associated cases. Of two subtype IV isolates, one was obtained from a non-disease associated case, and the other one from a patient with unknown status. For two subtype V isolates, data concerning *M. kansasii* disease were unavailable.

The highest frequency of *M. kansasii* subtype I isolations was observed for Poland (140/142; 98.6%), and the lowest for Estonia (2/7; 28.6%).

This study demonstrated that subtype I represented the vast majority of *M. kansasii* clinical isolates worldwide. Since all *M. kansasii* subtypes detected (I-VI) were isolated from both disease-related and non-related cases, subtyping of the species does not permit differentiation between disease and non-disease states that did and did not cause definite disease. Furthermore, the genetic diversity of the *M. kansasii* population showed important regional variations.

The study was performed within the framework of the Fight Against Tuberculosis in Central & Eastern Europe consortium (https://fate-consortium.org).

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GENETIC DIVERSITY OF MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN PAKISTAN

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Tuberculosis (TB) remains an inglorious leader among infectious diseases in mortality, with its annual toll of 1.7 million lives worldwide. Pakistan ranks 5th among the world's highest TB burden countries and the 6th among countries with the highest burden of drug-resistant TB, including multi-drug resistant (MDR)-TB. However, very limited data are available on the genetic structure of *M. tuberculosis* strains circulating in this country.

The objective of this study was to explore the genetic diversity of multidrug-resistant *M. tuberculosis* isolates from Pakistan with two different methodologies, i.e. spoligotyping and 24-loci MIRU-VNTR typing.

The study included 130 MDR-TB isolates, recovered from as many patients from Pakistan, between January 2013 and June 2015. Conventional drug susceptibility testing was performed using the standard 1% proportion method on the Löwenstein-Jensen medium, as described elsewhere. Spoligotyping was performed with a commercially available kit (Mapmygenome India Ltd., Madhapur, India) according to the manufacturer's protocol. MIRU-VNTR analysis was carried out at 24 loci, as described earlier. Phylogenetic clades of *M. tuberculosis* were assigned according to signatures provided in the SITVIT database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE).

Spoligotypes were obtained for 127 (97.8%) isolates. Based on a SIT number in the SITVIT database, all isolates presented 53 different profiles split into 14 clusters (n = 88, 69.3%, 2–30 isolates per cluster) and 39 (30.7%) unique patterns. MIRU-VNTR typing identified 128 unique types (98.5%) and one cluster (n = 2, 1.5%). When spoligotyping and MIRU-VNTR typing was used in combination, only two, out of 130 isolates, clustered both in both methods, resulting in a clustering rate of 1.5%.

Upon phylogenetic analysis, 101 (77.7%) isolates were classified into 12 clades, with the most prevalent being CAS1_DELHI (n = 53, 41.7%) followed by T1 (n = 14, 11%) and BEIJING (n = 10, 7.8%). The remaining 9 families (CAS, MANU2, EAI5, T2, LAM10_CAM, H1, X1, H4 and CAS2) involved 24 (18.9%) isolates. Twenty-six (20.5%) isolates could not be assigned to any specific lineage.

This study provides a snapshot of the genetic diversity of *M. tuberculosis* strains circulating in Pakistan. The compactness of the drug resistant *M. tuberculosis* population structure was apparent, as three major lineages, i.e. CAS1_DELHI, T1, and BEIJING comprised more than half (60.6%) of the isolates studied. Furthermore, the exceptionally low clustering rate

Suggest that recent transmission does not play an important role in the incidence of MDR-TB in Pakistan.

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UPDATE ON VIRULENCE FACTORS IN MYCOBACTERIA

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Although the majority of mycobacteria represent harmless environmental bacteria, a few mycobacterial species have evolved into major human pathogens. *Mycobacterium tuberculosis*, the etiological agent of human tuberculosis, is the most dominant mycobacterial pathogen in terms of global patient numbers and gravity of disease.

The molecular mechanisms by which *M. tuberculosis* induces disease are complex and result from a long-lasting host-pathogen co-evolution that might have started already by its *Mycobacterium canettii*-like progenitors. Recent research has revealed numerous factors implicated in the pathogenesis of *M. tuberculosis*, although the pathogen still holds many secrets of its successful strategy to circumvent host defences and persist in the host. As many pathogenicity factors relate to the exchange and secretion of biomolecules by *M. tuberculosis*, special emphasis is given to secretion pathways that enable *M. tuberculosis* to circumvent immune defence mechanisms mounted by the host. These factors might represent new, alternative targets for development of combination therapies that would enhance the efficacy of the immune system in controlling *M. tuberculosis* infections. Similarly, selected secretion systems may also represent important virulence factors in selected non-tuberculous mycobacteria. Here, recent insights into evolution of selected factors of *M. tuberculosis* and selected other mycobacteria that are involved in host-pathogen interaction will be discussed.

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GENOTYPING OF MULTIDRUG AND PRE-EXTENSIVELY DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM A HIGH INCIDENCE TB AREA IN MOROCCO

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The emergence of extensively drug-resistant tuberculosis (XDR-TB) has raised public Health concern for global control of TB. Although drug resistance-associated mutations in multidrug-resistant *Mycobacterium tuberculosis* complex (MTBC) isolates in Morocco were characterized, mutations in Pre-XDR/XDR isolates and their genotypes have not been reported previously. Resistance to second line antituberculosis drugs (SLDs) is mainly due to mutations in specific genes: *gyrA* and *gyrB* for resistance to fluoroquinolones (FQs), *rrs*, *eis* and *tlyA* for resistance to injectable drugs (kanamycin (KAN), amikacin (AMK), and capreomycin (CAP).

A collection of 70 MTB isolates already characterized as MDR and 100 pan-susceptible isolates randomly selected from a high incidence TB area in Morocco were enrolled in this retrospective study. The mutation profiles associated with resistance to SLDs: FQs and injectable drugs were assessed by DNA sequencing. Target sequences for four genes were examined: *gyrA* and *gyrB* (FQs), and *rrs* (KAN, AMK, and CAP) and *tlyA* (CAP). The fingerprint of each isolates was established by spoligotyping and 15-loci MIRU-VNTR typing methods.

Molecular analysis showed that 26.7% of MDR isolates are pre-XDR strains harboring mutations in *gyrA* gene. The most prevalent mutation involved in FQ resistance was Asp94Gly (50%). None of the isolates harbored mutations neither in *gyrB* nor in *rrs and tlyA* genes. Likewise, none of the pan-susceptible isolates displayed mutations in targeted genes. Spoligotyping of MDR MTB isolates resulted in 4 and 5 orphan and unique patterns respectively, and 61 strains in 9 clusters (2–26 strains per cluster) with a resulting clustering rate and recent transmission rate of 87.1% and 74.3% respectively. The most prevalent spoligotype was SIT42 (LAM; 37% of isolates). The repartition of strains according to major MTBC clades was as follows LAM (46.1%) > Haarlem (59%) > ill-defined T superfamily (17%) Haarlem (7%) > clade S (6%) > Beijing (3%) > T2-T3 and Beijing-like (1%). Of note, CAS (Central Asian) and EAI (East-African Indian) strains were absent in this setting. Subsequent 15-Loci MIRU typing failed to find any cluster of SIT/MIT, all clusters established by spoligotyping were splited, 70 unique MLVA-MtbC15 profiles were generated with a resulting clustering and recent transmission rate equal to zero meaning that all MDR strains are not a part of an established transmission chain and that the developpement of drug resistance in this setting is likely a result of inadequate treatment rather than primary resistance. HGDI analysis of the 15 MIRU loci showed that loci 10, 40 and Mtub04 were highly discriminative in our setting. All pre-XDR isolates harboring mutations in *gyrA* gene had not specific/paticular pattern generated by any of the two methods.

This study provides a first global snapshot of MDR MTBC population structure in Morocco. The results obtained (i) highlight the need for rapid detection of mutations associated with resistance to SLDs in order to adjust timely the treatment and to interrupt the propagation of virtually untreatable form of the disease, (ii) confirm that TB in Morocco is almost exclusively transmitted through evolutionary-modern MTBC lineages belonging to principal genetic groups 2 and 3 (Haarlem, LAM, T), with extremely high level of biodiversity seen by 15-MIRU typing, (iii) validate the use of spoligotyping in conjunction with 15-MIRU-VNTR scheme for future investigations in Morocco that should ideally use modified 15-loci MIRU-VNTRs (to include MIRU 23 instead of MIRU16), (iv) confirm the use of both the two typing methods to understand the transmission dynamic of tuberculosis in this setting.

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THE CORRELATION BETWEEN LEVELS OF PHENOTYPIC RESISTANCE AND GENOTYPIC MUTATIONS OF *M. TUBERCULOSIS*

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Rapid identification of drug resistance of *Mycobacterium tuberculosis* allows an earlier initiation of an adequate treatment regimen that potentially can reduce TB morbidity, mortality and transmission. New diagnostic methods have provided a promising solution for rapid and reliable detection of drug resistant TB. Despite the fact that rapid molecular assays are less accurate than the culture-based methods and raise the possibility of false negative results, the molecular characterization of the resistance spectrum of *M. tuberculosis* isolates offers the opportunity for overcoming the phenotypically detected resistance. So far, mutations within the *rpoB* gene confers a different level of phenotypic resistance for rifampicin (RIF) as well as mutation in *inhA* is link with low resistance to isoniazid (INH).

Objective was to study the correlation between phenotypic and genotypic resistance of *M. tuberculosis* on different levels of drug concentrations.

The genotypic resistance profiles for isoniazid and rifampicin of *M. tuberculosis* sputum isolates were assess by MTBDR*plus* and where correlated with culture based (MGIT-960) drug sensitivity test results. The different level of inhibitory concentrations of rifampicin and isoniazid of individual strains, assessed by MGIT-960 equipped with EpiCenter TB eXiST, were correlate correspondingly with the mutation types in the *rpoB* gene, and the presence of *inhA* mutation in the same *M. tuberculosis* isolates.

The *M. tuberculosis* isolates from 4568 patients with pulmonary tuberculosis were assess. 64.2% of them were INH resistant, and in 1,9% (n = 86) of these isolates, resistance was conferred by *inhA* mutation only. RIF resistance was detected in 61.9% of subjects, and in 27.2% (n = 762) of these the mutation *rpoB531* was missing. 30.6% of INH resistant *M. tuberculosis* strains, conferred by inhA mutation only and 28.6% of RIF resistant *M. tuberculosis* strains without S531 mutation, were sensitive to high concentrations of drugs by phenotypic DST.

The correlation of genotypic tests results with phenotypic resistance levels can be crucial forward a personalized approach in TB patient treatment, stopping the spread of drug resistance and promotion of the optimum use of the few drugs available for resistant TB treatment.

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GENOMIC EPIDEMIOLOGY OF TUBERCULOSIS: FROM WITHIN HOST EVOLUTION TO GLOBAL MIGRATION PATTERNS

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The availability of thousands of *Mycobacterium tuberculosis* genomes allows not only to infer how the pathogen emerged and spread but also to identify specific loci associated. I will present our work using evolutionary analyses to generate a high-resolution picture of the emergence, global spread and local transmission of the pathogen as well as genomic determinants associated. I will show how non-selective processes like genetic drift contribute to the genomic diversity of the pathogen with downstream consequence at the transcription and methylation levels. In the second part of the talk I will also discuss different approaches to identify and track new drug resistance determinants using a combination of functional genomics and genomic diversity analyses from different countries and from different patients through time and space. Overall our analyses reveal new bacterial factors associated to virulence and drug resistance. I will show however how the frequency of genetic variants associated to different traits, even if advantageous, depend on the conditions of local TB control more than on the high fitness of the bacterial genotype.

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EMERGENCE OF BEDAQUILINE RESISTANCE AFTER COMPLETION OF BEDAQUILINE-BASED DRUG-RESISTANT TB TREATMENT: A CASE STUDY FROM SOUTH AFRICA

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Treatment outcomes for drug-resistant tuberculosis (DR-TB) are poor with only 52% of MDR-TB and 24% of XDR-TB patients successfully treated. To address the global DR-TB epidemic WHO has released guidelines for the use of bedaquiline (BDQ) for the treatment of rifampicin-resistant or MDR-TB for specific indications. However, standardised methods to perform drug susceptibility testing (DST) have not been defined and BDQ resistance mechanisms remain poorly characterised.

Illumina NextSeq whole genome sequencing (WGS) was used to characterise serial *Mycobacterium tuberculosis* (Mtb) isolates from a patient receiving BDQ in Khayelitsha, South Africa. Phenotypic drug susceptibility testing (DST) for BDQ was performed in MGIT-960 media (concentration 1 μg/ml).

WGS showed an initial infection with a strain resistant to 7 drugs (rifampicin, isoniazid (low-level), ethambutol, ethionamide, fluoroquinolones, pyrazinamide and streptomycin). Following initial treatment failure with a standardised MDR-TB regimen, the patient was placed on a regimen containing 6 effective drugs (including BDQ, based on WGS). Isolates taken prior to BDQ initiation were BDQ-susceptible (phenotypically). WGS of subsequent serial isolates revealed the acquisition of a variant in *Rv0678* (conferring BDQ-resistance) one month after stopping BDQ treatment. Subsequent isolates showed the loss and gain of several other *Rv0678* variants, with only one variant (138 G insertion) fixed in the last available isolate. All isolates with *Rv0678* variants were BDQ-resistant.

The systematic gain and loss of *Rv0678* variants in isolates taken after completion of BDQ-based treatment illustrates the complex ongoing evolution patterns of *M. tuberculosis* as the concentration of BDQ decreases in the patient (long half-life). An alternative explanation is the emergence of existing BDQ-resistant Mtb from lesions which rupture following continuation of treatment without BDQ and after stopping all TB treatment. The emergence of BDQ resistant *M. tuberculosis* following stopping of treatment poses a risk of transmission of BDQ resistant clones to close contacts. Monitoring of pre-existing and emerging BDQ resistance should be a priority for all routine use and should continue post BDQ cessation.

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WHOLE GENOME SEQUENCING SHEDS LIGHT ON THE TRANSMISSION DYNAMICS OF A MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* OUTBREAK OVER 23 YEARS IN A HIGH INCIDENCE SETTING

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Whole genome sequencing (WGS) has shown that *Mycobacterium tuberculosis* strains are more genetically diverse than previously assumed and that traditional genotyping methods cannot discriminate strain heterogeneity with high resolution, which may mask their ability to accurately define the directionality of an outbreak, particularly in high tuberculosis (TB) incidence settings.

The objective of this study was to examine the evolution of a single *M. tuberculosis* cluster defined by a particular IS*6110* RFLP pattern to understand transmission and strain diversity over time.

Clinical *M. tuberculosis* isolates (n = 97) with identical IS*6110* RFLP fingerprint patterns were selected from a longitudinal sample bank of *M. tuberculosis* isolates collected from a high TB incidence suburb in the Western Cape, South Africa from 1993–2015. DNA was extracted from *M. tuberculosis* cultures for WGS and subsequent analysis. Available WGS of *M. tuberculosis* isolates from surrounding suburbs were screened and additional isolates

from the same time period with the same genotype were included in the phylogenomic analysis. The genomic variants identified by WGS were used for phylogenomic inference, drug resistance prediction and to determine genomic distances between isolates.

WGS analysis revealed unexpected genomic diversity within the seemingly homogenous IS*6110* cluster of *M. tuberculosis* isolates. Despite the IS*6110* RFLP based uniformity, at least six non-time dependent sub-clusters and several orphan-isolates were evident from the WGS-based phylogeny and genomic comparisons. Sub-clusters gained drug resistance conferring mutations (beyond MDR) on multiple occasions and *M. tuberculosis* isolates from surrounding suburbs were observed throughout the phylogeny.

IS*6110* RFLP typing underestimated the complexity of this 23-year outbreak. This study suggests that there is continuous circulation and reintroduction of this *M. tuberculosis* cluster in the community setting. Even with the advent of the WGS-era, confirming direct epidemiological links or outbreak directionality remains a challenge in high TB burden, low-income settings.

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A 15-YEAR SPATIOTEMPORAL ANALYSIS OF *MYCOBACTERIUM TUBERCULOSIS* LINEAGES 1 AND 2 IN CHIANG RAI, THAILAND

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Chiang Rai is the northernmost province of Thailand with high burden of tuberculosis (TB) and high TB death rate. Chiang Rai consists of various ethnic groups in three different geographic areas including (1) the bordering area in the northern and northeastern, (2) the central area, and (3) the outlying districts in the southeastern, southern and southwestern.

We aimed to assess the spatial and temporal distribution of *Mycobacterium tuberculosis* (MTB) lineages in the three different areas over 15 years.

Whole genome sequence (WGS) data was used to classify the genotypes of 1497 MTB using lineage-specific single nucleotide polymorphisms (SNPs). The spatiotemporal distribution of MTB lineage was analyzed in the three different areas during early 2000s (2002–2006), late 2000s (2007–2011) and 2010s (2014–2018). Stoddart and Taylor's index (G) was calculated to determine genotypic diversity of MTB lineage in the different settings.

In 2000s, lineage 2 (East Asian) was a highly predominant genotype (45%) in Chiang Rai followed by lineage 1 (Indo-oceanic) (41%). In 2010s, lineage 1 became the most dominant genotype (51%) replacing lineage 2 (35%). The overall change in predominant lineage from lineage 2 to lineage 1 was caused by a dramatic increase proportion of lineage 1 in the central area (from 44 to 53%) and in the outlying districts (from 39 to 59%). In the bordering area, a combined impact of increasing distribution of lineage 1 (from 32 to 40%) and other lineages (from 18 to 24%) was an additional cause of changing predominant lineage. The Stoddart and Tayloy's analysis of genotypic diversity showed that the central area had the highest diversity of MTB lineage (G = 4.14±0.46) followed by the bordering area (G = 3.67±0.76) and the outlying districts (G = 3.63±0.62).

Our study combining genotypic and space-time analysis has revealed a dynamic population changes in MTB lineage over 15-year period in Chiang Rai. Further studies on social determinants and patient's demographic data in the different geographic areas have the potential to provide effective TB control in the different setting.

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MOLECULAR-GENETIC METHODS OF DETECTION OF TUBERCULOSIS AND ITS DRUG RESISTANCE IN ARKHANGELSK REGION IN 2017

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Burden of tuberculosis (TB) is decreasing in Arkhangelsk region in northwestern Russia with incidence declining from 45.9/100 000 in 2012 to 21.6/100 000 in 2017 in civil society (excluding penitentiary society). Introduction of molecular-genetic tests of detection of TB and its drug resistance (DR), including multidrug-resistant (MDR) and extensively drug resistant (XDR) TB, is one of the key components of regional TB program. It plays an important role in improvement of diagnostics and management of TB patients in the region.

The objective was to evaluate performance of molecular-genetic tests used for detection of TB and its DR among patients with TB registered in civil society in Arkhangelsk region in 2017.

Line probe assay (LPA) (Hain Lifescience, Germany) and GeneXpert MTB/RIF (Cepheid, USA) were used for detection of *M. tuberculosis* (MTB) and its DR alongside conventional sputum smear microscopy (SM) and culture. All patients were initially tested with SM followed by LPA (Genotype MTBDR*plus*) if result of SM was positive or GeneXpert if result was negative using the same sample. In case of DR to isoniazid and/or rifampicin, Genotype MTBDR*sl* was performed to detect additional DR to fluoroquinolones and injectables, including XDR. In cases suggestive of nontuberculous mycobacteria (NTM), SM or culture positive with negative results of LPA or GeneXpert for MBT, identification was performed using Genotype Mycobacterium CM/AS.

Total of 214 "new cases" and 28 "relapses" of TB were registered in Arkhangelsk region in 2017. MTB was detected in 160 (74.8%) out of 214 "new cases" and all of them were tested for DR using molecular-genetic tests. 53 patients (31.1%) had MDR-TB, among them 3 patients had additional DR to injectables and 2 patients had XDR-TB. Among 28 "relapses" MTB was detected in 24 (85.7%) patients. 13 patients (54.2%) had MDR-TB, among them in 1 patient additional DR to injectables and in 2 patients to fluoroquinolones was detected. NTM associated disease was diagnosed in 4 patients (2 — *M. avium*, 1 — *M. gordonae*, 1 — *M. interjectum*).

All TB patients in Arkhangelsk region were tested with molecular-genetic tests before the treatment enabling quicker diagnostics and earlier treatment initiation. Early diagnosis ensures proper treatment regimen for patients with TB and NTM diseases. As a result management of TB patients is improved leading to better treatment outcomes and subsequently reduced TB transmission in the region.

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DETECTION OF EXTRACELLULAR *MYCOBACTERIUM TUBERCULOSIS* SMALL RNAs

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According to WHO, tuberculosis infection is one of the top ten deadly infections in the world, and about one-fourth of the human population is a carrier of latent tuberculosis infection (LTBI) without manifestation of disease symptoms. The LTBI associated with the conversion of *M. tuberculosis* bacilli to a dormant, metabolically inactive state; however, the molecular mechanisms of this change is not well studied. In many researches the small RNAs (sRNAs) was proposed as regulators of these processes. The aim of the study was detection of sRNA transcripts in cultural supernatant of *M. tuberculosis* strain H37Rv and into the blood serum of mice (C57Bl) infected with LTBI.

Mycobacterial cells were grown in Middlebrook 7H9 containing 10% ADC supplement at 37°C and harvested at different growth phase for use. The culture of *M. tuberculosis* was centrifuged at 6000g for 20 min at 4°C. The supernatant was filtered through 0.22 μm filters to remove the remaining bacteria. Bacterial total RNA was extracted from *M. tuberculosis* cultural supernatant by ExtractRNA reagent (Evrogen, Moscow, Russia), followed by digestion with Turbo DNase-free kit (Ambion, Austin, TX, USA) to remove contaminating DNA. cDNA was synthesized with 1.5 μg of total RNA by M-MLV (Evrogen, Moscow, Russia) transcriptase and random hexadeoxynucleotides according to the manufacture's instruction. The quantitative RT-PCR (qPCR) was carried out with the qPCRmix-HS SYBR (Evrogen, Moscow, Russia) and the CFX-96 real-time PCR detection system (BioRad, USA). All primers used in this study were designed using VectorNTI 11 (Invirogen, USA) and GeneRunner software (http://www.generunner.net) and synthesized at Evrogen (Moscow, Russia). LTBI C57Bl mouse model was designed as previously described (Shramko et al., 2010). The certain sRNA transcripts have been detected in *M. tuberculosis* culture supernatant at different growth phases (exponential phase, stationary phase and late-stationary phase) and into the blood serum of mice infected with LTBI. Obtained data allow us to propose *M. tuberculosis* sRNAs as new markers for LTBI diagnostic in the future.

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TB PORTALS PROGRAM: DATA-DRIVEN MULTINATIONAL CONSORTIUM AGAINST DRUG-RESISTANT TUBERCULOSIS

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TB Portals Program (TBPP) is an international initiative that is founded, developed and steered by doctors, researchers, IT specialists and radiologists to combat drug-resistant tuberculosis (DR-TB). The efforts of hospitals and biomedical research institutes from ten countries on three continents are organized and supported by TBPP to actively establish and grow an integrated open-access network of innovative tools and data from real patient cases of TB (tbportals.nid.nih.gov). The TB Portals collect, analyze, standardize, and present anonymized clinical, laboratory, and socioeconomic data, full bacterial genomes, and radiological data (CXR and CT).

The TBPP database currently has more than 1300 published (22 250 total cases), 75% of which are MDR or XDR-TB. Clinicians supply and validate all patient data. Once validated, the data become published with open access status in accordance with NIH FAIR principles. Importantly, both original and derived (expert-based and computational annotations) data remain patient-centric, i.e. linked to a unique patient identifier. This cornerstone principle enables users to define and analyze cohorts of patients, augmenting OMICS studies with diverse clinical information.

To date the database contains 730 published (1300 total) fully sequenced and annotated *Mycobacterium tuberculosis* genomes associated with the patient case record. We will highlight several TBPP projects studying 1) genomic signatures for TB relapse and reinfection, and 2) comparative analysis of *M. tuberculosis* strains isolated from sputum vs. surgically removed parts of lungs.

TBPP assists doctors and researchers in testing and refining their hypotheses with friendly and powerful tools. Starting from genomics and the molecular basis of drug resistance, we will demonstrate how our online tools enable anyone to simultaneously look at clinical, microbiological and radiological evidences in order to (1) search for genomic clues for inconsistencies in existing DR-TB diagnostics and to (2) study common and countries-specific evolutionary patterns of *M. tuberculosis.*

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SIMPLIFYING NGS APPROACHES TO OPTIMIZE TRACING OF TRANSBORDER SPREAD OF *MYCOBACTERIUM TUBERCULOSIS*

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Molecular epidemiology, and more recently genomic epidemiology, based on whole genome sequencing (WGS), improve our understanding about the transmission dynamics of *Mycobacterium tuberculosis* in a population. However, in many countries, including many of those with a high burden of TB, systematic genomic epidemiology cannot be implemented. Trying to find a solution to this situation, we propose an alternative line of progression, which tries to conciliate the discriminatory power of WGS with the speed, low cost and simplicity of PCR-based approaches. The cost of this shortcut is that it sacrifices the complete knowledge of all the transmission clusters in a population, because it needs to focus on surveying the strains that deserve special attention because they are more actively transmitted, or correspond to high-risk MDR or XDR strains. This short-cut strategy has proved to be efficient to survey actively transmitted strains, to fast track outbreak-strains, to update the presence of high-risk strains in a population or to give an urgent answer to public-health alerts, such as to rule out secondary cases due to the importation of XDR-TB cases. More recently, we are integrating this strategy to optimize the characteriza-

tion and tracking of trans-national transmission events. We have activated a decentralized multinational network of surveillance nodes. This network simultaneously analyze the cross-border distribution of relevant strains by means of sharing the same set of strain-specific PCRs. Once new cases infected by the surveyed strains are captured by the strain-specific PCRs the isolates are characterized by WGS. From these data we to define in detail the network of relationships between the involved cases. It allows us to differentiate transmissions after arrival of migrants to the host countries from independent importations from their countries of origin. Integrative multinational efforts supported on novel simplified strategies can transform the way in which we survey TB transmission in a new global scenario.

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GENETIC DIVERSITY AND DRUG RESISTANCE OF *MYCOBACTERIUM AVIUM* IN ITALY

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Mycobacterium avium complex is responsible for most of the human-associated nontuberculous mycobacteria infections. *M. avium* is classified into 4 subspecies, each endowed with specific pathogenetic and host range characteristics; among these, *M. avium* subsp. *hominissuis* (MAH), that is usually isolated from human and swine sources, is an important pathogen that causes infections in the respiratory tract, lymph node, and, occasionally, soft tissue of immunocompetent patients; moreover, it causes disseminated diseases in patients with human immunodeficiency virus infection. In Italy, as in many other countries worldwide, MAH is the most common cause of nontuberculous mycobacteria infection and the incidence of MAH infections is increasing. In the present study, we determined the VNTR-based genetic diversity of a collection of 71 MAH human strains isolated from 2010 to 2016 in order to estimate the genetic relationships among MAH isolates in our setting. Moreover, we performed the clarithromycin susceptibility test in order to investigate whether there was any association between the VNTR pattern and the minimal inhibitory concentration (MIC) of clarithromycin. The VNTR analysis revealed 24 distinct VNTR patterns; of these, 16 patterns were unique, while 8 patterns were shared by 2 or more isolates, thus yielding 8 lusters including a total of 55 isolates. Our results showed that most MAH isolates displayed a close genetic relationship, indicating that the MAH genotypes are quite homogeneous in our geographical area. Such genotypic stability of the MAH strain population circulating in our region supports the hypothesis of the presence of possible local sources of infection and transmission pathways at the local level.

Clarithromycin showed strong antimicrobial activity against MAH isolates, as indicated by the high proportion (94.4%) of susceptible strains. No significant association between VNTR genotype and MIC of clarithromycin was found; moreover, due to the small number of resistant isolates, it was not easy to evaluate the correlation between VNTR genotypes and clarithromycin susceptibility.

Further investigations on larger collections of MAH strains of human, animal and environmental origin, are needed both to define the correlation between genotypes and clinical features or drug resistance and to clarify the sources of infection and the specific transmission pathways of our region, in order to achieve a better control of MAH infection.

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GENOTYPES OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM DIFFERENT ORGANS OF PATIENTS WITH GENERALIZED TB AND HIV-COINFECTION

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The purpose of the study was to genotype *Mycobacterium tuberculosis* isolates from internal organs of patients from St. Petersburg, Russia, with generalized tuberculosis (TB) and HIV coinfection, to assess possible association between the *M. tuberculosis* genotype and localization of TB disease.

A total of 128 strains of *M. tuberculosis* were recovered from 55 patients with HIV infection of stages 4 or 5 and generalized TB with multiple lesions of internal organs (from 2 to 10), were studied. Most of the patients had affected lungs (50 cases), intrathoracic and intra-abdominal lymph nodes (46 and 34 cases respectively), spleen (40 cases), kidneys (32 cases), brain and meninges (32 cases). *M. tuberculosis* isolates were cultured from lungs (48), intrathoracic and intra-abdominal lymph nodes (40), spleen (20), kidneys (14), meninges and brain (7). *M. tuberculosis* were isolated from one affected organ in 11 patients, and in the remaining cases isolates from 2 or 3 affected organs (16 and 28 persons, respectively) were obtained. Genotyping was performed by spoligotyping (all strains) and IS6110-RFLP typing (Beijing genotype), the obtained profiles were compared with the international database SITVIT_WEB and a proprietary database (Narvskaya, 2003), respectively. The data were subjected to statistical analysis.

67.3% (37 of 55) patients were infected by *M. tuberculosis* Beijing genotype. Of the 37 patients infected with Beijing strains, strains of cluster A0 were isolated from 14 patients, B0 from 5 patients, and 10 other RFLP types were obtained in the remaining 18 patients. The non-Beijing genotypes were represented by LAM (4), Ural (5), T (5) and 4 others (1 strain of each genotype), all of which belong to the Euro-American lineage of *M. tuberculosis* (lineage 4). No difference was observed for isolates from different organs of the same patient. Of the 37 patients infected with Beijing strains, the lungs were affected in 33 patients, intrathoracic lymph nodes in 28, spleen in 28, intra-abdominal lymph nodes in 28, kidneys in 24, and brain and meninges in 24 patients. Of 18 patients infected with non-Beijing strains, lungs were affected in 17 patients, intrathoracic lymph nodes in 18, spleen in 12, intraabdominal lymph nodes in 11, kidneys in 8, brain and meninges in 10. Comparison of different organs for association with infection by Beijing and non-Beijing strains did not reveal statistically significant differences: lungs ($P = 0.890$); intrathoracic lymph nodes ($P = 0.503$); spleen ($P = 0.777$); intra-abdominal lymph nodes ($P = 0.970$); kidneys ($P = 0.448$); brain and meninges ($P = 0.886$).

Beijing genotype was predominant among *M. tuberculosis* isolates studied. Among Beijing family strains, cluster A0 (corresponding to the Central Asian Russian strain) prevailed. There were no statistically significant differences between Beijing and any of non-Beijing genotypes with regard to frequency of isolation from particular organs.

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PHYSIOLOGICAL IMPACT OF THE EVOLUTION OF THE *rpoB* MUTATION

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Bacilli within an infected lung cavitary lesion spontaneously evolve mutations that confer resistance and are subsequently selected following antibiotic treatment. During this evolutionary process both drug susceptible and drug resistant bacilli may be present. This mix state of susceptive and resistant bacilli captured at a distinct point in time may change during the course of infection and drug selection. The complexity of the population structure in each sputum sample may thus define the outcome of molecular and phenotypic drug resistance testing which in turn may determine how the patient will be treated. We hypothesize that the *rpoB* mutation will influence the transcriptome of the rifampicin mono-resistant isolate compared to the progenitor rifampicin susceptible isolate.

A sputum sample from an individual patient containing a heterogeneous population of both a rifampicin mono-resistant Beijing Ser531Leu clone and its susceptible progenitor was selected. DNA was extracted and sequenced using the Illumina HiSeq platform and analyzed using an in-house bioinformatic pipeline. RNA was extracted and sequenced using the Illumina platform and analyzed using Chipster, an open source bioinformatic platform.

The small number of variants between the two isolates suggests that the resistant isolate evolved from the susceptible progenitor. Our comparative transcriptomic analysis showed that microevolutionary events within the *rpoB* gene had a considerable influence on transcription. Consequently, the expression of bacilli's stress response, sigma factors, and regulatory genes were down regulated. This in turn led to a down-regulation of expression of a large number of genes, suggesting that the rifampicin resistant mutant has an altered physiology.

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MYCOBACTERIUM TUBERCULOSIS DRUG RESISTANCE MUTATIONS AND UNDERSTANDING OF PK/PD: TREATMENT AND CARE IMPLICATIONS

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Russian Federation has the third-highest burden of multidrug-resistant tuberculosis (MDR-TB) in the world and complicated by high rates of human immunodeficiency virus (HIV) co-infection which leads to mortality and risk for acquired *Mycobacterium tuberculosis* drug resistance. Treatment outcomes may be a consequence of pharmacokinetic/pharmacodynamics (PK/PD) variability.

In Irkutsk, we aimed to describe pharmacokinetic variability, minimum inhibitory concentrations (MICs) for key anti-TB drugs and their molecular correlates of resistance, and to determine if PK/PD variability associates with treatment response.

Consecutive people living with HIV initiating TB treatment at Irkutsk Regional TB Referral Hospital were recruited. After 2 weeks of treatment, medications were directly administered and plasma samples collected at 2 and 6 hours after administration. Drug concentrations were measured using validated liquid chromatography-mass spectrometry assays for peak concentration (C_{max}), the highest value in the dosing interval, and area under the concentration curve from time 0 to 6 hours (AUC_{0-6}). *M. tuberculosis* MIC testing was performed using the MYCOTB Sensititre plate. A drug was classified as active when C_{max} was greater than MIC. PK/PD variability as a predictor of treatment outcome was determined by classification and regression tree (CART).

69 patients with HIV had PK/PD testing. Mean age was 34 years (SD±6.2), 45 (65.2%) were male. Mean CD4 count was 180 (±202) cells/mL. Thirty-six (52.2%) had drug susceptible TB, 10 (14.5%) MDR-TB, 17 (24.6%) pre-extensively drug-resistant (XDR)-TB and 6 (8.7%) with XDR-TB. Based on PK/PD testing, patients were treated with a lower number of active drugs (3.25±1.40) compared to the number presumed to be active when initially prescribed (4.81±0.94), $p \leq 0.001$. Fifty patients had treatment outcomes and 16 (32.0%) had treatment failure. In CART analysis, regardless of molecular mutation for drug resistance, having less than 4.5 active drugs as redefined by PK/PD testing, correctly identified 15 of 16 (93%) of patients with treatment failure.

In Irkutsk, PK/PD testing predicted treatment outcome for patients with HIV/TB. Screening for mutations in *M. tuberculosis* resistance determining regions is an important method for constructing initial regimens, but should be followed by PK/PD testing to attain the highest likelihood of drug activity.

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GENOMICS AND LOCAL ADAPTATION OF *MYCOBACTERIUM AVIUM*

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Mycobacterium avium subsp. *hominissuis* (MAH) is a human pathogen that causes *M. avium* complex (MAC) lung disease, which is difficult to cure by current antibiotics treatment. It has been suggested that MAH circulates between the human body and the environment. Despite its clinical significance, the genetic mechanisms underlying local adaptation of this pathogen are unknown due to a lack of population-wide genomic data. To overcome this issue, we evaluated the genetic population structure of MAH using genome-scale data from 36 global strains (including 12 Japanese strains sequenced in this study), and then sought to identify alleles unique to Asian populations by comparative genomic analysis. The population structure analysis was extended to include 652 global strains using the multiple-locus variable-number tandem repeats data set, which revealed that two genetic population groups dominated the Asian isolates.

By analyzing mutual homologous recombination and gene content, we revealed that MAH reproduces sexually and has an unlimited gene repertoire. The results of these analyses predict the presence of a chromosome

exchange mechanism called "distributive conjugative transfer (DCT)," recently discovered in other mycobacterial species (Gray et al., Science. 2016. 354: 347–350). We also identified chromosomal loci where allelic variation was correlated with geographic origin. One noteworthy discovery was that the origin of genes responsible for trehalose biosynthesis differed between Asian and other MAH populations. Furthermore, we observed transmission of alleles encoding "mammalian cell entry proteins" between East Asian populations. Therefore, sexual reproduction, likely via DCT, plays a critical role in local adaptation of MAH.

As a new concept, we present a model for the life cycle of *M. avium*, in which *M. avium* generates progeny with diverse genomes via "mating" in a common environmental pool, prior to infection of human hosts. After infection, the progeny is subjected to natural selection within the host, followed by the re-release of clones with adaptive alleles into the environment. This concept may also be relevant for other mycobacterial species.

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UTILITY OF WHOLE GENOME SEQUENCING (WGS) OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ISOLATES IN PRACTISE

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Since 2016, all culture positive *M. tuberculosis* complex isolates have been subjected to WGS to allow comparison with the current laboratory diagnosis in the Netherlands. The utility of WGS was investigated for 1) identification of (sub) species and genotypes; 2) drug susceptibility testing; and 3) investigation on tuberculosis transmission.

The new SNP-IT (SNPs to identify TB) method, developed at the RIVM/the Netherlands and Oxford University/the UK, traces SNPs shared exclusively by members of each (sub) species, lineage, and sub-lineage. The total number of unique SNPs identified ranged from 23 for *M. bovis* to 6.837 for *M. canettii*. The SNP-IT method was applied to 1.157 routine samples from 2016/2017 in the Netherlands and compared with identification by Reverse Line Blot, PhyResSe, and Coll SNP-barcode. This comparison showed that SNP-IT more accurately identifies all animal (sub) species and is more specific in identifying sub-lineages of lineage 4. A small proportion (n = 176) of lineage 4 isolates could not be identified by SNP-IT due to high similarity to the H37Rv reference genome; these are in the Coll SNP-barcode system identified as lineage 4.5/4.7/4.8.

Phenotypic drug susceptibility testing (MGIT) was compared with the detection of resistance-associated mutations by WGS for first-line antibiotics rifampicin, isoniazid, ethambutol, and pyrazinamide. In total, 1.134 isolates from 2016/2017 in the Netherlands were included. For all drugs, the negative predictive value (NPV) was > 99%. In general, rifampicin and isoniazid had most optimal scores. For rifampicin, the sensitivity was 100%, specificity 99.8%, the positive predictive value 95%, and the NPV 100%. This was 98%, 99.2%, 92.5%, and 99.8%, respectively, for isoniazid. WGS was also able to predict intermediate/low level resistance for rifampicin, isoniazid, and pyrazinamide. A minority of isolates showed discrepancy between MGIT and WGS results; these isolates are re-tested to explain discrepancy results.

Both VNTR typing and WGS were applied to all isolates from 2016. In total, 535 isolates were genotyped, of which 25% (134/535) were clustered by VNTR and 15% (82/535) by WGS. The proportion of identified epi-links among WGS clustered cases (50%) was much higher than among VNTR clustered cases (31%). This study was repeated with isolates from 2016 and 2017 to analyse transmission over two years.

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MINOR GENETIC DETERMINANTS OF SECOND-LINE INJECTION DRUGS RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

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Second-line injection drugs group, which include kanamycin (KAN), capreomycin (CAP), and amikacin (AM), is one of the cornerstones used in the treatment of MDR tuberculosis. Though the main resistance mechanism leading to cross-resistance to all three drugs described in *Mycobacterium tuberculosis* is the alteration of 16S rRNA, other mechanisms also could be found in clinical strains, such as promoter mutations of the *eis* and *whiB7* genes leading to KAN resistance, and TlyA inactivating mutations leading to CAP resistance. In consequence, a noticeable number of resistant strains do not carry any known mutations.

We performed the next-generation sequence analysis of the 5 Beijing and one Haarlem lineage clinical *M. tuberculosis* strains with discordant results of phenotypic resistance to injection drugs, and genetic analysis of *rrs*, *eis*, *tlyA*, and *whiB7* loci. The sequencing data were analyzed using the Galaxy web platform (http://usegalaxy.org). Further bioinformatic analysis of the obtained SNPs was performed with custom Python scripts and public databases ReSeqTB and PolyTB.

We found 2126–2229 SNPs for Beijing lineage and 1606 SNPs for Haarlem lineage isolates compared to the referent H37Rv strain. Upon the exclusion of known mutations associated with resistance, fitness compensation and deep bioinformatic analysis, the list of candidate SNPs, potentially associated with resistance, was shortened to 10–100 for each strain. The novel putative mechanisms of resistance included mutations in elongation factor EF-G, phosphotransferase Aph, hypothetical protein Rv0147, secretion protein EspG2, and aspartate aminotransferase AspC.

The diversity of drug resistance mechanisms reflects the complexity of microevolution of *M. tuberculosis* and impacts the sensitivity of molecular tests. Improvement of our knowledge of drug-resistance mechanisms would facilitate the discovery of new drugs together with the prediction of drugs interactions and promote the development of molecular assays.

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PECULIARITIES OF THE TUBERCULOSIS, HIV AND HIV-ASSOCIATED TUBERCULOSIS INCIDENCE IN THE FAR EASTERN FEDERAL DISTRICT OF THE RUSSIAN FEDERATION

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Objective of the research was to analyze the dynamics of the tuberculosis (TB), HIV and HIV-associated TB incidence in the Far Eastern Federal District (FEFD) of the Russian Federation.

The data of the official statistical forms No. 8, No. 33, No. 61 was used. Modern statistical methods were applied.

The TB incidence declined by 43.2% (from 85.1 to 48.3‰) during 2008–2017 in the Russian Federation when prevalence declined by 42.2% (from 190.7 to 109.8‰). The most pressing tuberculosis epidemiological situation persisted in the FEFD. During a decline of TB-incidence in the FEFD in the years of 2009–2017 by 32.9% the regional index was still higher compared to the Russian-wide rate by 1.8 — 1.8 times. HIV-infection in 2017 reached 36.0‰ versus 14.0‰ registered in 2008 in the FEFD. During the analyzed period HIV-incidence accession rate in the FEFD constituent entities changed repeatedly. In 2017 the HIV prevalence rate in FEFD was lower than the Russian-wide index by 2.2 times. The highest HIV prevalence in the FEFD was registered in the Primorsky, Sakhalin Territories and the Chukotka Autonomous Region (57.3; 50.9 и 50.0‰ respectively). The HIV-TB associated incidence in the Russian Federation reached 8.3‰ in 2017 versus 4.3‰ in 2009. During the analyzed period of time percent of the HIV-positive patients with associated TB increased from 6.4 to 17.2% in the Russian Federation. In the FEFD the same index raised from 2.0 to 4.8%. This said the Russian-wide index consistently exceeded the FEFD rate by 3.1–4.6 times. The highest accession rates of the present index in the FEFD were registered in the Khabarovsk Territory — 9.9%, the Amur region — 9.4%, the Republic Sakha (Yakutia) — 6.6%.

The FEFD is a leading territory of TB-incidence for several years. An increase in the number of HIV-positive people as well as HIV-TB co-infected patients was registered in the region. Substantial differences of the analyzed indices in different constituent entities of the FEFD were registered.

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GLOBAL WHO POLICIES ON MOLECULAR METHODS FOR TB DIAGNOSIS

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The World Health Organization's (WHO) global strategy for TB prevention, care and control for 2015–2035 (known as the End TB strategy) calls for the early diagnosis of TB and universal drug susceptibility testing (DST), highlighting the critical role of laboratories for rapidly and accurately detecting TB and drug resistance. This requires ensuring access to WHO-recommended rapid diagnostics and universal access to drug-susceptibility testing (DST) for all patients with signs and symptoms of TB. WHO defines universal access to DST as rapid DST for at least rifampicin among all patients with bacteriologically confirmed TB, and further DST for at least fluoroquinolones among all TB patients with rifampicin resistance. The WHO estimates that 10.4 million persons developed tuberculosis (TB) worldwide in 2016, including 490 000 cases of multidrug-resistant TB (MDR-TB) and 110 000 cases of rifampicin-resistant TB (RR-TB), needing the same second-line treatment regimen.

The objective of the study was to present WHO policies on molecular methods for TB diagnosis.

Xpert MTB/RIF and/or Ultra are recommended as initial test for diagnosis of all persons with signs and symptoms of TB. Line probe assays are recommended as rapid diagnostic tests for detection of resistance to Isoniazid, Rifampicin, Fluoroquinolones and Amikacin. DNA Sequencing is becoming increasingly important as a reference method for detecting mutations associated with resistance to first and second line anti-TB drugs.

According to WHO policies, molecular methods play critical role in global fight with TB.

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PREVALENCE OF NONTUBERCULOUS MYCOBACTERIUM spp. STRAINS ISOLATED FROM CLINICAL SPECIMENS AT NORTH ESTONIA MEDICAL CENTRE IN 2001–2017

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Mycobacteria are widespread microbes in nature. Mycobacteria that are not causative agents of human and animal tuberculosis are designated as non-tuberculous mycobacteria (NTM). NTM caused infections start to become more frequent in the recent years. This report summarizes data of the NTM isolates reported at North Estonia Medical Centre in the period 2001–2017. In Estonia, physicians are not requested to report infections involving NTM species. Reports of the prevalence of MTBC are available in the Estonian Tuberculosis Registry. In contrast, reporting of NTM suspected to be involved in a disease, does not represent the absolute occurence and distribution of the NTM species and disease. These reports are not verified and any association with clinical data should be interpreted with caution.

The incidence of tuberculosis in Estonia shows a trend of decrease. The aim of the study was to analyse the prevalence of NTM strains on the basis of clinical specimens.

The NTM species were isolated from clinical material that was sent to the mycobacteriology laboratory for diagnostic purposes during 2001–2015. The pathological material was decontaminated by NaOH+NALC and cultured on Löwenstein–Jensen and Middlebrook media. The NTM species were identified by the GenoType Mycobacterium CM/AS test (Hain Lifescience GmbH).

In 2001–2017, of 114 907 investigated specimens, 12 790 (10.9%) MTBC and 1020 (1.04%) NTM strains were identified. The leading NTM species isolated were *Mycobacterium avium* (41.7%), *M. gordonae* (17.5%), *M. fortuitum* (9.3%) and *M. intracellulare* (6.8%). The rarely isolated NTM were represented by *M. kansasii*, *M. xenopi*, *M. szulgai* and *M. abscessus*. Of the NTM, 72% were cultured from sputum, 5% from blood and 23% from other materials. In total, 85 patients were infected with NTM in 2001–2005, 210 in 2006–2010 and 331 in 2011–2017. Of the NTM infected patients, 35% were > 65 years old and 9% were < 30 years old. A TB and HIV coinfection was found in 317 (8.3%) and NTM-HIV coinfection was found in 56 (10.5%) patients.

During the last 17 years the prevalence of NTM in patients' material has increased approximately three times. The most prevalent species is *M. avium*.

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THE INFLUENCE OF THE H2 COMPLEX ON *MYCOBACTERIUM AVIUM* INFECTION IN MICE

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Mycobacterium avium is the opportunistic pathogen in humans, animals and birds and the most common cause of non-tuberculous mycobacterial lung infections worldwide. Analogously to other mycobacterial infections, its antigens are presented predominantly in the context of the Class II *MHC* molecules resulting in activation of $CD4^+$ T cells producing IFNγ, the key cytokine in antimycobacterial response and infection control.

Addressing genetic control of *M. avium*-triggered disease, we compared two congenic strains of mice on the B6 genetic background established in our lab — B6.I-100 ($H2\text{-}A^jE^j$) and B6.I-139 ($H2\text{-}A^{jb}E^j$) — that carry different alleles encoding the β-chain of the *H2-A* gene. After aerosol *M. avium* challenge, B6.I-139 mice died earlier and displayed more severe cachexia compared to B6.I-100 mice. Measurement of the CFU counts in lungs and spleens at weeks 8, 12 and 18 post infection, revealed significant differences in the lung phenotype at the early phase (more CFUs in the lungs of B6.I-100 mice). Assessment of lung pathology demonstrated diffuse inflammation in the lung tissue of B6.I-139 mice at week 8 post infection and granulomata containing foamy macrophages and necrotic zones during the chronic phase. Flow cytometry and immunohistochemical staining revealed higher neutrophil inflammation in the lungs of B6.I-100 mice, accompanied by an increased expression of genes involved in neutrophil attraction. The level of proinflammatory TNFα, but not IL-6 and anti-inflammatory IL-10 and TGF-β, was higher in the lungs of B6.I-100 at an early stage of infection. Importantly, lung $CD4^+$ T cells from more resistant B6.I-100 mice were more activated ($CD44^{hi}CD62L^{lo}$ phenotype) and produced significantly more IFNγ in response to mycobacterial antigens during chronic stage of infection. Higher numbers of lung $CD4^+$ T cells in B6.I-139 mice in 8 weeks after challenge may reflect an attempt of the host to control infection early after challenge, apparently not successful. Of note, it is not clear whether interstrain differences in disease progression reflect differences in the efficacy of antigen presentation between $H2\text{-}A^b$ alleles and subsequent T cell activation, or T cell exhaustion during chronic stage of the immune response. Overall, our data suggest that the allelic differences in the H2-A molecule are involved, albeit moderately, in control to *M. avium* infection.

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MYCOBACTERIUM AVIUM-TRIGGERED DISEASE: HOST GENETICS AND IMMUNITY IN MOUSE MODELS

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Mice of the I/St strain are extremely susceptible to *Mycobacterium tuberculosis* but resistant to *M. avium* infection, whereas B6 mice show a reversed pattern of susceptibility. By directly comparing: (i) characteristics of susceptibility to two infections *in vivo* (ii) architecture of lung granulomata and (iii) expression of genes encoding regulatory factors of neutrophil influx in the lung tissue, we demonstrate that genetic susceptibility of the host determines the pattern of lung pathology. *M. avium*-infected B6 mice and *M. tuberculosis*-infected I/St mice are prone to develop necrotizing granuloma surrounded by hypoxic zones, massive neutrophil influx and B-cell follicles in the lung tissue. These mirror-type lung tissue responses demonstrate that the level of genetic susceptibility of the host to a given mycobacterial species largely determines characteristics of pathology, and emphasize the importance of host genetics in pathogenesis. Segregation genetic analysis and development of novel H2-recombinant congenic strains allowed dissection of genetic control of two infections. Regarding susceptibility to and severity of *M. avium*-triggered disease, involvement of two distinct genes was clearly demonstrated: the *Slc11a1* (former *Nramp1*) gene, acting as a major genetic factor, and the classic Class II *MHC* gene *H2-Ab*, a minor modifier of susceptibility pattern.

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EVOLUTION AND TRANSMISSION OF *MYCOBACTERIUM TUBERCULOSIS* RESISTANCE TO FLUOROQUINOLONES

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Fluoroquinolones (FQs) have been widely used for the tuberculosis (TB) treatment for decades and *Mycobacterium tuberculosis* strains resistant to FQs have been reported globally. In the past few years, we had gained some insights into the evolution and transmission of *M. tuberculosis* FQ-resistance. Firstly, we found that FQ-resistance mostly appeared in multi-drug resistant (MDR) *M. tuberculosis* strains. We observed the emergence and transmission of FQ-resistance in clinical clustered (as defined by whole-genome sequencing) MDR cases and we speculated that the general inclusion of FQs in the first-line treatment regimen in western China may contribute to the high resistance rate among MDR cases. By studying the within-host heterogeneity of *M. tuberculosis*, we proved that the evolution of FQ-resistance is associated with the emergence and competition of several resistance related mutations in DNA gyrase genes, a process for selecting highly resistant and low-cost strains. Lastly, we studied the mechanisms of primary ofloxacin-resistant strains to acquire resistance to moxifloxacin (new generation FQ) and we found a secondary mutation in DNA gyrase associated with this process.

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EPIDEMIOLOGY OF EXTRAPULMONARY TUBERCULOSIS IN ALBANIA, 2010–2016

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Extrapulmonary tuberculosis (EPTB) is a therapeutic challenge. Possible reasons include ist under- and overdiagnosis/reporting. Here, we report results of the cross-sectional retrospective review of the epidemiology of EPTB in Albania from 2010 to 2016.

The objectives of the study were to find out epidemiological characteristics of EPTB and to explore risk factors, and challenges in the diagnosis and management of EPTB in Albania.

We used data from National TB Program and included all cases of TB diagnosed in the Albania from 2010 to 2016. Information on age, sex, year of diagnosis, and anatomic location of the site of disease was retrieved from central database of National TB Program.

In Albania during 2010–2016, 925 cases of extrapulmonary TB were reported, males were 581 (63%) and females 344 (37%). The number of cases diagnosed per year was as follows: 170 (38.2%) in 2010, 129 (30%) in 2011, 108 (25.7%) in 2012, 141 (29.7%) in 2013, 147 (36%) in 2014, 117(28.2%) in 2015 and 113 (27.2%) in 2016.

Sputum smear examination, X-ray and culture examination and tissue biopsy were carried out in 58; 42.3; 18 and 15% of patients respectively for EPTB diagnosis. The most affected age group was < 65 years (23%). Pleural effusion (35%) and lymph node (15.7%) were the most common types of extrapulmonary TB.

Patients live in urban areas (60%) rather than rural (40%). The mean age of EPTB patients is 44.5 and pulmonary TB patients is 41.2. Incidence of EPTB decreased from 5.5/100 000 in 2010 to 5.1/100 000 in 2016.

In Albania, extrapulmonary TB in 2010–2016 showed a slight decrease in incidence, although the rates are still very high. Diagnosis of extrapulmonary TB was made according to national guidelines, however long delay has been reported in most cases before the final diagnosis. Microbiological proof is the key to diagnosis and treatment, and tissue biopsy that should be required regularly.

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DETERMINANTS OF TB RELATED DEATH FROM TUBERCULOSIS PATIENTS IN THE NORTHERN THAILAND

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Tuberculosis is the most common cause of deaths from respiratory infection in Thailand. Understanding the risk of death could provide useful information to provide better clinical care for TB patients. *N-Acetyltransferase 2 (NAT2)* gene is the main determinant of isoniazid (INH) metabolism. *NAT2* rapid acetylator contributes to lower anti-TB drug (INH) serum concentration and increased risk of treatment failure and relapse from INH based TB regimens.

The aim of this study was to determine the effect of *NAT2* acetylator status on TB related death.

TB patients were recruited from the TB registry during 2002–2011 in Chiang Rai province, Thailand. The *NAT2* acetylators (rapid, intermediate and slow) were determined by haplotype specific polymerase chain reactions, HS-PCR. These groups of patients were excluded from further analysis: 1) patients who did not receive the INH based regimens for TB treatment; 2) patients who did not receive the INH based regimens longer than 2 weeks and 3) patients who died within the first 2 weeks of TB treatment. Mortality-associated risk factors within 1 year of treatment were analyzed using Cox-regression model.

Of 1.076 TB patients who met study criteria, 213 (19.8%), 495 (46.0%) and 368 (34.2%) belonged to *NAT2* rapid, intermediate and slow acetylate group respectively. In total, 115 patients died within 1-year follow-up. In the multivariate analysis, rapid *NAT2* acetylator status increased the risk of death when compared against *NAT2* intermediate acetylator (adjusted hazard ratio [aHR]: 1.83, 95%CI: 1.15–2.91). The effect of *NAT2* rapid acetylator on deaths is more significant in HIV positive TB patients (aHR 2.68, 95%CI:1.14–6.26). The risk factors associated with death was different among the *NAT2* acetylators. In *NAT2* rapid acetylator group, elderly people, HIV positive, past TB history and smoking status was increased the risk of death.

The *NAT2* rapid acetylator is related to the mortality during TB treatment. The inadequate treatment with the doses of standard regimens caused by *NAT2* rapid acetylator might increase the risk of death in TB patients, these NAT2 pharmacogenetic risks are interacting with other clinical risk factors, which is depended on the acetylator status.

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CLICHES AND DOGMAS IN MOLECULAR TUBERCULOSIS RESEARCH

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I will present a personal critical view on some hot issues of the molecular epidemiology of tuberculosis, in particular, regarding an uncritical use of some well known online tools and resources. Invaluable for establishing terminology and classification in molecular epidemiological studies of *Mycobacterium tuberculosis*, they are limited by our insufficient knowledge of genome evolution and uncritical perception of their indications. This is exemplified by partly inadequate (sub)clade assignment due to imperfect decision rules, and misleading methodological approach when scientifically unsound phylogenies are built from spoligotyping data.

To begin with, I propose the following definitions. First, "molecular mythology" that relies on minimal array of references that conveniently support long-lasting clichés. Second, "molecular iconography" that relies on dogmatic perception of the current knowledge when online databases are uncritically regarded as ideal icons. Finally, I introduce the term "click science". In contrast to the fascinating and sophisticated click languages, "click science" relies on uncritical and simplified perception of knowledge and a dogmatic, iconographic view of indications provided by increasingly convenient online tools and databases. For example, spolTools is an example of the click phylogenetics when a plethora of statistics is generated in few clicks but their exploration is minimal. In its turn, click systematics is exemplified by SITVIT's (i) reader-unfriendly huge tables with different possible percentages and (ii) easy to read but partly inadequate (sub)clade labels.

Labels are convenient for classification, but should be revisited in the context of modern knowledge. The "we have been taught this way" approach reflects the mentality of a conservative teacher rather than a creative researcher. As Heidegger once said, "knowledge does not think"; indeed why think when it already knows? As far as science is concerned, this quotation from Henry Gee's "The accidental species" is much more appropriate: "Science is about neither Facts nor Truth, but the quantification of doubt".

Below are examples of some clichés pertaining in molecular epidemiology of tuberculosis.

Firstly, pathogenic properties of the Beijing genotype are traditionally listed as increased virulence, association

with drug-resistance, high transmissibility and clustering. But they do vary among endemic and sporadic strains, in different settings and hosts, even at within country level.

Secondly, Russian epidemic clone Beijing B0/W148 was commonly regarded as widespread across all Former Soviet Union. In reality, its geographic distribution shows a peculiar clinal gradient with highest frequency in Siberia and sharp decrease in the Asian part of the former Soviet Union.

Thirdly, a global spread of LAM RD-Rio sublineage has been claimed and was attributed to its particular pathogenic properties. A comprehensive analysis of available data shows that RD-Rio strains are rarely present in Russia and East Asia. It appears that there is no global dissemination of RD-Rio due to specific virulence properties of these strains but rather their spread due to human migration (if such migration did take place).

A confusing terminology, misclassification and false clustering are not abstract issues but make a scientific discussion meaningless, and I will propose some courses for improvement of the situation.

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ANALYSIS OF SECONDARY RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* TO SECOND-LINE ANTI-TUBERCULOSIS DRUGS IN CASABLANCA

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Tuberculosis (TB) is a major public health problem in Morocco, despite multiple strategies and funds allocated. According to epidemiological data, with about 31.452 TB cases detected in 2016, and a national incidence of about 91 per 100 000 inhabitants per year, TB pose a serious threat to TB management in Morocco, with Casablanca being one of the most affected region.

The objective of this retrospective study was to evaluate secondary resistance of *Mycobacterium tuberculosis* to 2nd line anti-tuberculosis drug in Casablanca.

In this retrospective study, 1300 patient samples from different CDTMRs and hospitals across Casablanca and regions over a 2-year period from January 1st, 2015 to December 31st, 2016 were analysed. Conventional techniques, such as BKD microscopic examination, BKC culture and antibiotic sensitivity were used for the diagnosis of TB.

Our results show that among the 1300 samples analyzed, 600 (46%) were found positive for MTB, of which 58.33% were male and 41.66% were female. Patients aged between 20 and 40 years was the most affected group, representing 78% of patients. Data using the conventional Petroff decontamination and homogenization technique for isolation, identification as well as titration of the "BK" strains were as follows: Negative culture (54%), Positive culture (46%). The antibiogram used for this study gave the following results: 53% were wild strains, 47% were mutants, among which 18% were "MDR" strains and 1% were "XDR" strains.

The results of the present study reflect the importance of a good management of TB cases in order to succeed the treatment regime adopted at the national level and the success of the fight against this scourge in Morocco.

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DEVELOPMENT OF THE EXTERNAL QUALITY ASSESSMENT SCHEME FOR NON-TUBERCULOUS MYCOBACTERIA DRUG SUSCEPTIBILITY TESTING IN EUROPEAN UNION

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Non-tuberculosis mycobacteria (NTM) are increasingly associated with pulmonary and extrapulmonary disease in humans. Although identification and drug susceptibility testing (DST) of NTMs comprises a significant part of the tuberculosis (TB) reference laboratory activities in the European Union (EU), currently no internationally recognised external quality assurance (EQA) schemes exist for NTM DST. It lacks standardization and evidence on how to interpret DST results for specific drugs is limited.

Recognising the need for harmonization of methodologies in EU/EEA, European Reference Laboratory Network for Tuberculosis (ERLTB-Net) in 2017 conducted a pilot study among National Reference Laboratories (NRL) aimed at understanding methods employed for identification and DST and developing an EQA scheme for NTM DST. Pilot study comprised a survey followed by an EQA round using identical panels comprising 10 well characterised rapid (*M. abscessus*) and slow (*M. avium*) isolates.

Completed questionnaires were received from a total of 32 NRLs (97.0% response rate). Thirty NRLs routinely perform identification of NTMs with a majority (77.4%; N = 24) using line probe assays as a primary means of NTM speciation. Reports containing minimum inhibitory concentrations (MICs) and result interpretation for five key drugs (Clarithromycin (CLA) Amikacin (AMK) Moxifloxacin (MOX); Linezolid (LIN), and Doxycycline (DOX) for *M. abscessus* only) were received from 21 NRLs (95.5% response rate).

Interlaboratory agreement rates were higher for *M. abscessus* isolates; all five strains were found to be resistant to DOX by all NRLs (MIC 8.0–16.0 μg/ml). Relatively minor variations were seen in MICs and their interpretations for MOX and AMK while for LIN MIC ranges for individual isolates varied greatly (2.0–32.0 μg/ml) across NRLs resulting in a lower agreement with regards to results interpretation. For slow growers AMK and MOX appeared to be the most problematic drugs both in terms of MIC determination (ranging 4.0–64.0 and 0.5–8 μg/ml in individual strains, respectively) and interpretation.

The results show that inter-laboratory reproducibility is insufficient, highlighting the need for expanding EQA schemes for NTM DST. As EQAs for *M. tuberculosis* complex DST have led to more reliable and reproducible phenotypic DST, we propose to follow a similar approach for clinically relevant NTM.

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POLYMICROBIAL BIOFILM FORMATION AS A POSSIBLE CAUSE OF UNEXPECTED DEFAULTED TREATMENT OF PULMONARY TUBERCULOSIS

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Microbes rarely exist as single species planktonic forms as they have been commonly studied in the laboratory. Instead, the vast majority exists as part of complex polymicrobial biofilm communities attached to host and environmental surfaces. *Mycobacterial tuberculosis* (MBT) is no exception. A number of researchers have shown that in the experiment *in vivo* model, MBT can form biofilm-like structures in the lungs.

The aim of the study is to demonstrate the role of tuberculomas satellite microbiota as example of polymicrobial biofilm existent in a lung of TB patients.

Our study of clinical MBT strains shown less 5% of them were able to produce mature biofilms (pellicle) on a liquid medium. Although we might expect that pathogenic MBT could gain obvious advantage in case of growth in necrotic foci in lungs and it should keep this ability in the first passage *in vitro*. It was found feature of MBT strains produced pellicle on liquid medium to grow on Levinshtein–Jensen by specific R colonies. It looks as disk with a convex center, "UFO-colonies". It was shown on *in vitro* model that about of 50% clinical MBT strains can coexist together with *Bacillus licheniformis*, also isolated from sputum of TB patient. Moreover, after pellicle formation by bacilli in the first 3 days, the growth of MBT was continued for next 30 days under the bacillary pellicle. It is very important that investigated bacilli had a high tolerance to streptomycin, ethionamide, isoniazid and ethambutol, e.i. to four of the 12 basic anti-TB drugs.

The study on 16S rRNA metagenomic and massively parallel sequencing (NGS) DNA of several tuberculomas was conducted. It was shown that quantity of MBT genomes were less 3% in all cases. The vast majority species belonged to Gram-positive *Firmicutes* like *Staphylococcaceae* and also a small amount of Gram-negative taxons was found.

We can assume that anti-tuberculosis therapy is confronted with not only MBT, but with polymicrobial biofilm communities, which formed by the etiological agents of tuberculosis and also by a large number of other satellite microorganisms in lungs. It is very important that this microbial community in TB-patient lungs of should form a cumulative resistance to anti-tuberculosis therapy during long-term treatment. We can expect that the cumulative resistance of a polymicrobial biofilm in the TB-patient lungs may be significantly differing from the resistance of detected in the clinical laboratory TB strains.

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BACTERIAL WGS AND HOST GENOME-WIDE SNP ANALYSIS OF TUBERCULOSIS PATIENTS IN THAILAND

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Mycobacterium tuberculosis has been a human pathogen for a long time, providing ample opportunities for genomic interactions between the two organisms. Evidences of co-evolution has been reported. We have performed genomic studies in a cohort of tuberculosis patients in Chiangrai, northern Thailand. The genomes of *M. tuberculosis* isolated from 1170 patients during 2003–2010 were sequenced. The genomes of the same patients were also evaluated using high-density SNP arrays. The bacteria were genetically heterogeneous, with majority belonging to various sublineages of lineages 1 and 2. Refinement of classification of lineage 1 were proposed and a few novel sublineages of the others were identified especially in remote populations. The patients mostly belonged to three genetic groups, identified by principal component analysis, and three self-identified ethnicity groups. The profiles of patients infected by sublineages varied especially among sublineages of lineage 2. There were strong correlations between the bacterial genotypes and human ethnicity. GWAS identified a few genes associated with particular genotypes of the bacteria. Together with historical records, this study indicated that both the founder effects and co-evolution may explain the associations. This study provided some insights to the bacterial host interactions and useful information for the development of vaccines and other control measures for tuberculosis and is being replicated in a cohort of 600 patients in 2016–2018 with some patients studied by WGS.

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POPULATION STRUCTURE OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM TB-HIV COINFECTED PATIENTS IN OMSK REGION, WEST SIBERIA, RUSSIA

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A clear trend of the increasing incidence of tuberculosis (TB) associated with HIV infection is observed in the Omsk region in West Siberia. The TB-HIV incidence increased from 0.3 in 2006 to 15.2 in 2017 per 100 000 population. The aim of this study was to analyze the population structure of *Mycobacterium tuberculosis* isolated from TB-HIV coinfected patients.

A total of 150 *M. tuberculosis* isolates were recovered from 150 patients with pulmonary tuberculosis in 2013–2017 were included in this study. They included 110 men (74.8%) and 40 women (25.2%), the average age was 35.2 years (from 22 to 58 years). *M. tuberculosis* culture and drug susceptibility testing were performed according to standard protocols. DNA was extracted from *M. tuberculosis* isolates using the recommended method. Beijing genotype was detected by PCR analysis of the *dnaA-dnaN*::IS*6110* insertion. Beijing B0/W148 cluster was identified by PCR analysis of the *Rv2664-Rv2665*::IS*6110* insertion. Spoligotyping was performed according to standard protocol (Kamerbeek et al., 1997) and the profiles were compared to SITVIT_WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE) for family assignment which was corrected by expert assessment. A chi-square test was used to detect any significant difference between the two groups.

Almost 3/4 of the studied *M. tuberculosis* isolates belonged to the Beijing genotype (109/150, 72.6%). Beijing B0/W148-cluster (Russian MDR Beijing clone) included 29 isolates (26.6% of Beijing population). Majority of the Beijing isolates (62/109; 56.8%) belonged to the Beijing 94-32-cluster (Central Asian/Russian strain).

Forty-three non-Beijing isolates were subdivided into 17 spoligotypes shared by 1 to 5 isolates. They represented the following genetic families: LAM (n = 19), T (n = 10), Ural (n = 6), Haarlem (n = 3), X (n = 1); for two isolates the family status was "unknown".

Population structure of *M. tuberculosis* isolates from TB-HIV coinfected patients in Omsk region is dominated by the Beijing genotype (72.6%) while the other, non-Beijing families belong to the Euro-American superlineage. Beijing genotype is dominated by the isolates of the epidemiologically important Beijing 94–32 cluster (56.8%).

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LOOKING INSIDE THE FOREST: FROM CLASSICAL GENOTYPING OF *MYCOBACTERIUM TUBERCULOSIS* TO WHOLE GENOME SEQUENCING IN HIGH MULTIDRUG RESISTANCE SETTINGS

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Molecular typing of *Mycobacterium tuberculosis* is an increasingly important public health tool that can provide a framework to investigate the dissemination and emergence of specific strains. Classical typing methods have relied upon the genetic analysis of repetitive loci, whose presence, number and layout on the *M. tuberculosis* genome have enabled the distinction between clinical isolates of different genotypes.

Over the last decade, the massive development of Next Generation Sequencing and ability to carry out Whole Genome Sequencing (WGS), which provides the ultimate resolution power, has revolutionized bacterial typing by enabling one to infer on the directionality of tuberculosis (TB) transmission. Herein, the importance of seeing deeper in the genome of *M. tuberculosis* will be analysed in two distinct epidemiological scenarios: the emergence of strains associated with drug resistance due to migratory movements and, the discrimination and study of the transmission dynamics of endemic multidrug and extensively drug resistant strains.

Regarding the emergence of drug resistant strains, WGS does provide sufficient evidence to delineate and discriminate within cross-border clusters that were otherwise impossible to discriminate. In Portugal, this has been of special relevance for multidrug resistant (MDR) superclusters of the Beijing family in Europe (such as the 94-32 and 100-32 types) that are spreading through vast geographical areas. This can be of great importance to inform concerted efforts aimed at screening migrant populations arriving from high-incidence settings and new epidemiological links can be uncovered even within the country. The same inability to discriminate using classical typing methods can be generated by outbreak strains whose circulation is occurring for decades. In such a scenario multiple transmission sub-clusters are usually present and WGS can effectively resolve these transmission networks. Good examples are the KZN, Lisboa or Q1 strains, all of which associated with extensively drug resistant (XDR) TB. Furthermore, recent evidence obtained by WGS shows that MDR-TB and XDR-TB within Lisboa and Q1 clades has emerged multiple times instead of more conservative predictions based on classical typing. Some roadblocks still lie ahead, but, the latter also highlights the advantage of genome-wide based phylogenetic analysis of *M. tuberculosis* clinical isolates in TB surveillance and, the need for a switch from classical typing to WGS-based typing.

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ADVANCES IN THE STUDY OF MOLECULAR BASIS OF RESISTANCE TO NEW ANTI-TB DRUGS

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Bedaquiline is an effective drug for the treatment of MDR and XDR tuberculosis allowing up to 85% cure rate in complex therapy. Unsuccessful treatment is accompanied with elevation of bedaquiline MIC and acquisition of mutations in *mmpR* and *atpE* genes. However, the clinical significance of mutations detection is still obscure due to an insufficient number of clinical isolates, characterized by phenotypic and molecular methods.

Bedaquiline MIC of clinical MTB isolates from patients, who obtain complex therapy including bedaquiline, were tested using both the agar proportion method on 7H11 plates and Bactec MGIT system. Genes *mmpR* and *atpE*, associated with an elevated MIC of bedaquiline, were sequenced.

191 clinical isolates were divided into several groups based on the genetic analysis: strains with wild-type sequences of all analyzed genes; heteroresistant strains, where both wild-type and mutant sequences could be identified; isolates where only mutant, or mix of different mutant sequences was found; and a group of isolates with the mutated *atpE* sequence. Most of the strains, isolated prior the bedaquiline treatment, had wild-type sequences and liquid media MICs ranged from 0.06 to 0.50 mkg/ml with the mode at 0.12 mkg/ml. Isolates with mutated *mmpR* gene possessed MIC range of 0.12–4.00 mkg/ml with mode at 0.25 mkg/ml. Heteroresistant isolates had an intermediate MICs from 0.12 to 2.00 mkg/ml. Four isolates with AtpE substitutions (D28N, A63P, A63V) had bedaquline MICs of 4.00 and 8.00 mkg/ml. The MICs distributions of wild-type and mutated isolates on 7H11 media had the distinct border between 0.06 mkg/ml and 0.12 mkg/ml: most of the strains with a MIC of ≥ 0.12 mkg/ml bore mutations.

During the treatment with bedaquiline, intermediate resistance emerged by selection of *mmpR* mutations, and high-level resistance caused by substitutions in AtpE. Our results also raise the question of reliability of currently used critical bedaquiline concentrations for 7H11 agar (0.25 mkg/ml) and Bactec MGIT 960 (1 mkg/ml) tests.

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THE IMPLEMENTATION OF NEXT-GENERATION SEQUENCING FOR EPIDEMIOLOGICAL STUDIES AND DRUG RESISTANCE INVESTIGATIONS IN MICRO-EPIDEMICS INVOLVING PEDIATRIC TUBERCULOSIS PATIENTS

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In Latvia, the childhood TB epidemiology trends very clearly reflected the increase of TB transmission from the year 1992 and the decrease of transmission rate since 2001. There was also a small increase of TB notification rate in children in 2011 which clearly predicted an increas-

ing incidence of adult TB cases in 2012, and was related to the economic crisis in Europe. The best strategy for TB case detection in children is contact investigation allowing early diagnosis, which, in turn, allows the implementation of the prophylactic treatment of TB infection, provides successful treatment outcomes, and prevents death. Current molecular diagnostic methods of *Mycobacterium tuberculosis* (Mbt) usually provide limited information that is often not sufficient for the local outbreak and transmission investigations. Implementation of the modern approaches such as Next generation sequencing technologies in the epidemiological studies of childhood TB has a potential to combine TB diagnosis, drug resistance profiling and epidemiological analysis into one test helping to initiate personalized treatment for every patient timely and correctly.

Case report. A patient, 29 years old, was diagnosed with the 3rd TB episode in her life in 2017. Mbt cultures were obtained and genotyped. Molecular genotyping results showed different spoligo and IS6110 RFLP patterns for all three TB episodes in years 2001, 2011, and 2017.

Epidemiological anamnesis revealed that the first TB episode at the 14 years of age in patient was identified in 2001 during household contact investigation — patient`s uncle was diagnosed with TB in 2001. Uncle had TB relapse in 2006. Genotyping results of the uncle`s both Mbt cultures obtained in 2001 and 2006, and patient`s Mbt culture obtained in 2011 revealed the identical spoligotype (SIT1) and IS*6110* pattern with 17 bands for both patients. These results indicated the high possibility of the transmission in the household contact. However, genotyping results from patient's Mbt culture obtained in 2017 showed different genotype.

Whole genome sequencing (WGS) was used for in-depth characterisation of *M. tuberculosis* isolates associated with matched pairs of TB cases. The obtained results were in accordance to the genotyping and drug resistance. In addition, the obtained data provided additional resolution of the microevolution of Mbt subpopulations.

The addition of WGS to the epidemiological data and social network analysis could improve the confirmation of the epidemiological links and evaluation of the transmission dynamics of TB. Additionally, rapid WGS data can be used to identify molecular evidence for strain-specific phenotypic variability including anti-mycobacterial drug resistance, further providing rapid onset of appropriate treatment.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN LATVIA

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Tuberculosis is still one of the major infectious diseases in Latvia, causing serious health problems. While the incidence of the disease has steadily declined in the country since year 2001, the rates of drug-resistant tuberculosis are among the highest within the European Union. Molecular genotyping of *M. tuberculosis* plays an important role both in clinical studies and in the epidemiological investigations, allowing to describe and characterize pathogen`s population structure. Our previous studies have shown that in Riga and Riga region the majority of *M. tuberculosis* isolates belonged to lineage 4 (Euro-American) and lineage 2 (East-Asia). The family distribution of the isolates comprised 25% Beijing, 27% T, and 25% LAM (Latin-American Mediterranean) isolates, while Haarlem, Ural, and X families were detected in 11, 6, and 3%, respectively. A high proportion of Beijing and LAM isolates is alarming, as these *M. tuberculosis* genotypes have been often associated with remarkable pathogenic features such as drug resistance and increased transmissibility. TB incidence in the Latvian region Latgale seems to be higher than the average, and in-depth studies of *M. tuberculosis* isolates in this region could provide additional resolution for the characterization of the lineages circulating in the country. The Latgale region borders with Lithuania in the South, Belarus in Southeast, and Russian Federation in the East. *M. tuberculosis* isolates in this region were studied by the Spoligotyping and IS*6110* RFLP genotyping methods. In total, 56 (73.7%) samples of 76 bacteriologically confirmed TB cases in the year 2017 were available for molecular analysis. The results showed that 52% of isolates could be classified as common genotypes in Latvia (SIT1, SIT42, SIT50, SIT53, SIT254, SIT262, SIT283, SIT1292), while 48% of isolates belonged to SITs which are rarely found in the country or were unique (SIT45, SIT47, SIT52, SIT65, SIT118, SIT150, SIT278, SIT1175, SIT1451). The most common spoligotype belonged to the T1 lineage (SIT53, 16%) followed by SIT1, SIT47 and SIT254 (9% each). Within all samples studied, 14 isolates (25%) formed 4 different clusters with 3–5 members in each. The epidemiological links were confirmed for nine patients in 3 clusters (SIT47, SIT65, and SIT1292). When the prevalence of different spoligotypes was analysed between different countries, a similarity between particular genotypes in Latvia and neighbouring countries was observed. In-depth analysis of these isolates on the international scale could be very useful in order to investigate the possible transmission dynamics of *M. tuberculosis* strains.

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FUNCTIONAL RELEVANCE OF *MYCOBACTERIUM TUBERCULOSIS* DIVERSITY: FROM GENOTYPES TO IMMUNE RESPONSES AND DISEASE SEVERITY

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The genetic diversity of tuberculosis (TB)-causing bacteria has surprised us over recent years. A growing body of evidence attributes a functional relevance to this diversity, both at the clinical and immune response levels. Investigating the full diversity of *Mycobacterium tuberculosis* in nature is however impossible. We recently moved from the study of limited collections of *M. tuberculosis* to an oriented approach, aimed at covering a representation of *M. tuberculosis* heterogeneity. For this, we studied over 600 TB patients in Porto and over 300 matching *M. tuberculosis* isolates. We show a highly homogeneous phylogenetic structure of *M. tuberculosis*, with nearly all cases belonging to Lineage 4 (L4). Within the L4 clade, the most represented sublineage was LAM. This host-pathogen sympatric distribution was however shak-

en by the presence of HIV or diabetes co-morbidities, which oriented the selection of 2 LAM *M. tuberculosis* isolates from TB patients with no comorbidities, but with different TB severities. Despite their close genetic structure, we are finding a distinctive pattern of cytokine production by human and mouse macrophages infected with either isolate. Interestingly, the high TB severity-associated isolate is a poor inducer of cytokine responses. Mechanistically, we relate this poor induction of cytokine production with a differential capacity of the bacteria in activating the host transcriptional machinery, as well as the inflammasome. Furthermore, a different *in vivo* progression of infection by the selected *M. tuberculosis* isolates was also observed. Most notably, the isolate associated with high TB severity showed higher dissemination patterns from the lung to the liver and spleen than that associated with mild TB. To investigate if the link TB severity-cytokine response was broader, we tested the cytokine response induced in human peripheral blood mononuclear cells by a series of other *M. tuberculosis* isolated from patients with different TB severities. A segregation of isolates was observed, with poor inducers of cytokine responses being generally associated with high TB severity. Collectively, our findings suggest that bacterial-intrinsic properties modulate the intensity of the initial immune response with likely consequences for the severity of TB. Our studies open interesting avenues for TB interventions, namely by raising the importance of considering the pathogen diversity when designing host-directed therapies.

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THE ROLE OF THE IS*6110* IN MICRO- AND MACROEVOLUTION OF *MYCOBACTERIUM TUBERCULOSIS* LINEAGE 2

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Genomes of *Mycobacterium tuberculosis* complex members contain the insertion sequence (IS) *6110* which, due to its high quantitative and positional variability, has become a widely used marker in epidemiological studies. The element plays an important role in microorganism genome plasticity, but still many consequences and causes of transposition have not been fully described. This work studies the transposition mechanism of IS*6110* and its impact on the evolution of *M. tuberculosis* (*Mtb*).

Whole-genome sequencing data of 902 *Mtb* lineage 2 isolates was obtained from NCBI and ENA databases. Phylogenetic sublineages were determined based on SNP analysis (120 samples belonged to the ancient Beijing (17 proto-Beijing, 28 Asia Ancestral 1, 13 Asia Ancestral 2, 38 Asia Ancestral 3), 782 samples belonged to the modern Beijing (10 Asian African 1, 29 Asian African 3, 65 Asian African 2, 43 Pacific RD150, 140 Europe/Russia W148 outbreak, 361 Central Asia) (E. Shitikov et al., SciRep, 2017). ISMapper was used to determine the sites of integration of the IS*6110* (Hawkey et al., BMC Genomics, 2015).

We obtained 17 972 points of insertion, which belonged to 865 independent positions in the H37Rv genome. The mean copy number per genome was 19.92 (from 9 to 25). To describe the evolution of an element in the genome, we arranged our samples in the order corresponding to a phylogenetic tree constructed on the basis of SNPs. We determined the stepwise mechanism of transposition, in which the transition to a new subpopulation is accompanied by a change in the localization of several copies of IS. It is important to note that the localization of the element in the ancestral population does not change, which implies a transposition only by "copy-paste" mechanism. In addition, we defined genes (537 sites (256 genes)) and intergenic regions (328 sites), where the element was integrated. Sixteen genes previously identified as being essential under different experimental conditions were found to contain IS. Further we carried out identification of IS*6110* mediated LSPs which showed the presence of recombination events (deletion) between inversely oriented elements.

In conclusion, we determined the evolution and role of IS*6110* for *Mtb* lineage 2 strains. We identified evolutionary and subpopulation-specific sites of integration which can be used for typing and subsequent research.

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NGS DETERMINATION OF MYCOBACTERIAL TRANSRENAL DNA AS POTENTIAL TOOL OF CLINICAL DIAGNOSTIC

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Sputum is a major object for monitoring TB treatment and diagnostics it is strictly depended from bacterial load. There is a substantial need for less variable and more reliable specimen for the diagnosis of tuberculosis and for treatment monitoring. The objective of this study is to estimation diagnostic power of full genome sequencing (NGS) of soluble mycobacterial transrenal DNA (mtr-DNA) in urine of TB patients and TaqMan tests designed after analysis of metagenomic data.

DNA patient with pulmonary tuberculosis (TB) isolated from 4 ml. of urine by QIAamp Circulating Nucleic Acid Kit. Detection of TB positive samples made by previously developed PCR targeted to 45 base pairs fragment of mycobacterial genome. It was chosen 2 positive PCR samples. It were mapped on the reference genome *M. tuberculosis* (NC_000962.3) by BWA. In total were mapped 16 579 paired reads of the one sample (0.83%) and 1 783 754 (64%) of the second sample respectively. There were also analyzed mapped DNA sequences with more than 4x coverage. The median length of mtr-DNA found as 20 bp.

It was found 156 mtr-DNA fragments repeated in both samples. The median length of DNA fragment was found was 20 bp. Five fragments including part of 16S rRNA gene were chosen for design primer and TaqMan probes for targets from 43 to 60 bp. Length of primers and probes were reduced by Locked Nucleic Acid (LNA) bases. The sensitivity and specificity of the developed tests was determined by known DNA samples from urine. The result obtained did not reveal a significant improvement in the sensitivity of the new tests. PCR-RT cutoff remained approximately 40 cycles, like in previously developed tests.

High specificity and sensitivity of NGS and low of PCR suggest that diagnosis and monitoring of tuberculosis by mtr-DNA should be based on NGS, rather than on PCR.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN KAZAKHSTAN, 2006–2018

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The implementation of various projects aiming to develop basis of molecular-epidemiological monitoring of tuberculosis infectious agent by combining scientific and technical potential of specialists from several research and medical centers resulted in extensive experience and contribution to understanding epidemic process of tuberculosis in Kazakhstan. In particular, we established an updated information database of genetic profiles using 24-MIRU-VNTR and spoligotyping, a set of reagents designed to carry out molecular profiling of mycobacterial isolates, as well as protocols based on reduced and expanded panels for stream high-throughput screening in 96- and 384-well format.

The studies were conducted on clinical isolates of *M. tuberculosis*, collected from 2006 to 2018 in hospitals of Kazakhstan. The samples were characterized by the resistance to first and second line antimicrobials using cultivation on Lowenstein–Jenssen media and BACTEC MGIT 960. Genotyping: manual 24MIRUVNTR-typing (Supply et al., 2006) and spolygotyping (Kamerbeek et al., 1994), compared with MIRUVNTR*plus* and SITVIT_WEB databases. Additional typing of hypervariable loci QUB-18, QUB3232, VNTR-3820, VNTR-4120 was used when insufficient genotypes' differentiation was observed (Iwamoto et al., 2007; AllixBeguec et al., 2014). Genetic polymorphism of drug resistance was determined by TB-TEST system (BIOCHIP-IMB, Russia), and GenoType MTBDRplus kits (Hain Lifescience, Germany).

Our pilot study of 2007–2008 established that Beijing strains present a special epidemic threat for Kazakhstan as they are the main reason for the majority of MDR tuberculosis cases and are widely distributed in different regions of Kazakhstan (Skiba et al., 2015). Further study (2012–2014, with 576 genotyped samples of drug-resistant strains) allowed not only to confirm the previous conclusions, but also clarified prevalence and impact of other genetic families in Kazakhstan. Both of these studies led to identification and confirmation of a separate genetic group, which we named KAZ-1.

These studies have shown some cases of tuberculosis caused by several mycobacterial strains simultaneously. We have also encountered genotype changes in patients during their hospital treatment. This finding formed the basis for the next project on the study of nosocomial transmission of drug-resistant tuberculosis (2015–2017). The result of this project was registration of several cases of genotype change in hospitalized patients at different treatment stages. This targeted monitoring also helped to record the first case of LAM RD-Rio strain in Kazakhstan.

The overall results inspired us to use the developed research algorithm that combines reduced VNTR panel with TB-TEST biochip system in the ongoing research to reveal the connection between drug-resistance mutations and strain genotype, and to better understand the epidemic process of tuberculosis in Kazakhstan.

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NEXT-GENERATION SEQUENCING OF DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS — FIRST SLOVENIAN EXPERIENCE

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Slovenia is low-incidence country with incidence rates of tuberculosis (TB) cases around 6.0 in last several years. In our country, the percentage of resistant TB is very low with sporadic cases of multidrug resistant (MDR) TB. The aim of our study was to examine the feasibility of a full-length gene analysis for the drug resistance related genes (*inhA, katG, rpoB, embB*) using Next-generation sequencing Ion Torrent technology and compare the results with those obtained from conventional phenotypic drug susceptibility testing (DST) in 61 TB isolates from our National mycobacterial culture collection. TB strains included were either susceptible or mono-, poly-, or multidrug resistant by phenotypic DST. High concordance between genetic (Ion Torrent technology) and standard phenotypic DST testing for isoniazid, rifampicin and ethambutol was observed with sensitivities of 68.2; 100 and 100%, and specificities of 100; 80 and 88.2%, respectively. In conclusion, the next-generation sequencing analysis successfully predicted drug resistance with significant shortening of time needed to obtain the resistance profiles from several weeks to just a few days.

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WGS IN ROUTINE DIAGNOSTICS OF TUBERCULOSIS — PREDICTION OF DRUG RESISTANCE AND GENOTYPING

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The aim of the study was to compare the performance of whole genome sequencing (WGS) and conventional assays for drug susceptibility testing and genotyping of *Mycobacterium tuberculosis* in a routine laboratory.

All *M. tuberculosis* cultures sent to the Finnish mycobacterial reference laboratory in 2014 were tested by Mycobacteria Growth Indicator Tube (MGIT) for first-line drug susceptibilities. Genotyping was performed by 24 loci MIRU-VNTR typing and spoligotyping. WGS was performed with the Illumina MiSeq system. For prediction of drug susceptibility, the data were analyzed using five software tools (PhyResSE, Mykrobe Predictor, TB Profiler, TGS-TB and KvarQ). Clustering analysis was performed using Ridom SeqSphere+ (Ridom GmbH, Germany) cgMLST v2 (2891 targets) for genomes assembled by Burrows-Wheeler Aligner (bwa). Isolates with allelic distance ≤ 12 formed a cgMLST cluster. In addition, SNP analysis using the GATK tools (Broad institute, Cambridge, MA, USA) was performed and clusters based on distance of ≤ 12 and ≤ 1 SNPs were formed.

The sensitivity of the five software tools to predict any resistance among strains was almost identical, ranging from 74% to 80%, and specificity was more than 95% for all software tools except for TGS-TB. The sensitivity and specificity to predict resistance to individual drugs varied considerably among the software tools.

Among the 211 isolates, 15 clusters comprising 36 isolates (19.9%) were found by conventional genotyping. Of these, 15 isolates (36.1%) were clustered similarly to six clusters also by cgMLST analysis. Furthermore, four strains that did not cluster by conventional genotyp-

ing clustered by cgMLST analysis resulting in 19 (9.0%) clustered isolates by cgMLST. By clustering analysis with the distance ≤ 12 SNPs, 18 isolates clustered into 7 clusters. With the 1 SNP cut-off, three clusters with a total of seven strains were found and these were similarly clustered also by cgMLST and conventional genotyping analysis.

A reliable prediction of drug susceptibility can be obtained with WGS combined with data analysis with software tools. In routine practice, *M. tuberculosis* isolates can be screened with WGS for mutations associated with drug resistance, and only resistant strains confirmed with the MGIT system. Compared to conventional genotyping methods, WGS analysis is more discriminatory, reducing the risk of false clustering and unnecessary contact tracing.

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SINGLE NUCLEOTIDE POLYMORPHISMS IN *hsp65* AND MACPPE12 GENES OF *MYCOBACTERIUM AVIUM* subsp. *HOMINISSUIS*

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Mycobacterium avium subsp. *hominissuis* (*MAH*) represents a group of environmental bacteria known as opportunistic pathogens of animals and humans, especially HIV positive. Polymorphisms in *hsp65* and MACPPE12 genes are used for identification, intra-subspecies differentiation and phylogenetic studies of *MAH* populations.

The aim of our study was to identify single-nucleotide polymorphisms (SNPs) in *hsp65* and MACPPE12 genes of clinical isolates from Russian patients with pulmonary and disseminated mycobacteriosis and to assess phylogenetic relationships of geographically distant *MAH* populations.

The sequence analysis of the 3'-portion of the *hsp65* gene and MACPPE12gene was applied for 40 MAH strains isolated from humans with mycobacteriosis (including 19HIV-positive) in St. Petersburg, Russia (2008–2011). The nucleotide sequences were aligned to the reference genome of *M. avium* subsp. *hominissuis* 104 (NC_008595.1.).

In total, the 40 *MAH* strains were classified into three different *hsp65* sequevars: code 1, code 2 and code 3. The majority of *MAH* strains (72.5%) belonged to code 1, the same sequevar as for *MAH* strain 104. The code 2 and code 3 included 3 (7.5%) and 8 (20%) strains, respectively. The largest *hsp65* sequevar code 1 has observed only in 4.7% of isolates from Japan and absent in Korean human isolates. The sequevars code 1 and code 2 predominated among *MAH* strains in the USA, Canada, Belgium.

The sequence analysis of the MACPPE12 gene revealed 20 SNPs grouped into nine sequevars at the nucleic acid level: NA01, NA02, NA03, NA06, NA10, NA13, NA14, NA19, and NA_Rus01. Among 20 SNPs eight were nonsynonymous resulting in seven sequevars at the amino acid level: AA01, AA02, AA04, AA07, AA08, AA13, and AA_Rus01. The sequevar AA02 consisted of three different NA variants with synonymous SNPs profiles: NA02, NA03, and NA06. Half of the *MAH* strains belonged to the sequevar AA02 (type NA02). The predominant cluster AA02 (type NA02)/code 1 and the unique variant AA_Rus01 (NA_Rus01) were identified among *MAH* strains from Russia. The present study demonstrated the prevalence of the sequevar AA02 in MAH strains isolated from humans in Russia, Japan, and Korea.

Thus, we confirmed the relative conservativeness of the nucleotide sequence of the *hsp65* gene but the polymorphism of the MACPPE12 gene. A comparative analysis of the SNPs profiles of the *hsp65* and MACPPE12 genes allowed to identify differences and similarities between geographically distant populations of *MAH*, which highlighted the variability of the global population of *M. avium* species.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN ALBANIA (2006–2011)

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Tuberculosis (TB) epidemics in Albania has been stable over the past years with a gradual decreasing incidence (from 18.7 to 14.8 per 100 000 inhabitants, in the period 2001–2016) with a slight deterioration in 2013 (16.8 per 100 000 inhabitants). First insight data (2008) on TB molecular epidemiology showed a moderate Recent Transmission Index (RTI) (28%) and a high level of genetic diversity.

We aimed with this study to better understand the correlation of ubiquitous and autochthonous *Mycobacterium tuberculosis* complex (MTBC) genotypes with available demographic and epidemiologic data over a six-year period, in Albania.

MTB strains isolated in Albania (n = 745, 1 isolate per patient) between 2006 and 2011 were analyzed by spoligotyping and MIRU-VNTR typing by 24 loci scheme. The data obtained were compared with MIRU-VNTRplus database. Using molecular typing 486 (65.23%) isolates (patients) were distributed into 113 clusters and the remaining 259 (34.77%) isolates had a unique pattern. The cluster sizes ranged from 2 to 21 isolates per cluster. RTI ((nc — c)/n) resulted 50.07%. The most predominant lineages were Ghana (28.59%), Haarlem (19.73%) UgandaI (18.79%), LAM (7.11%), Ural (5.64%), TUR (3.89%) and Caprae (3.49%). Other lineages identified were Cameroon (1.74%), X (1.48%), S (1.21%), Bovis (0.54%), Delhi/CAS (0.54%, Beijing (0.4%) and West African 2 (0.13%). This study highlighted the predominance of five shared spoligotypes: ST 53 (T1) (n = 166, 22.28%), ST 4 (LAM3 and S/convergent) (n = 39, 5.23%) and ST 42 (LAM9) (n = 38, 5.10%) ST 613(T1) (n = 37, 4.97%) and ST 47 (H1) (n = 35, 4.70%). Of the unknown spoligotype signatures three were more frequent than others (4.70%, 3.22%, 3.22%), their origin and historical link to other genotypes is yet unknown. Among the MLVA MtbC15-9 types, MLVA 15411-85 and MLVA 15419-69 (both of unknown spoligotype signatures) resulted the predominant types involved in recent transmission in Albania (two biggest clusters identified with 21 and 19 identical isolates respectively).

In conclusion, MTBC genetic population in Albania is highly heterogenous. TB epidemics in Albania is fueled mostly by evolutionary-recent lineages. It is largely dedicated to recent transmission (50.07%). Autochthonous genotypes result linked to the 2 biggest clusters identified. One of them is found exclusively in Tirana (MLVA 15411–85). The new MTBC genotypes will require further molecular characterization.

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THE IMPACT OF THE DELETION OF THE MMP-1 GENE ON THE EXPRESSION OF SYMPTOMS AND THE EFFECTIVENESS OF TREATMENT IN PATIENTS WITH PULMONARY TUBERCULOSIS

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The objective of the study is to examine the impact of the deletion of the MMP-1 gene on the development, expression, dynamics of clinical syndromes and the effectiveness of treatment in patients with pulmonary tuberculosis.

73 patients with pulmonary tuberculosis, receiving treatment in the hospital of the state health care facility "Regional TB Dispensary" in the city of Astrakhan. Depending on the genotype of MMP-1, three groups were formed: the first group — with G1/G1 — 15 people (20.5%), the second — with G2/G1 — 27 people (50.7%) and the third one — with G2/G2 — 21 people (28.8%).

During the treatment, the disappearance of symptoms of intoxication and bronchopulmonary disorders in patients of the 1st and 2nd groups occurred earlier than in the 3rd group ($\chi^2 = 11.5$, $p = 0.02$). Dissolving of infiltration in the lung tissue also started earlier in the first and second groups ($\chi^2 = 9.2$, $p < 0.05$). The discharge of *Mycobacterium tuberculosis* (MBT) stopped earlier in patients of the 1st group. After four months of treatment, MBT were not determined by any method in 94.6% of the patients in the 2nd group and in 90.4% in the 3rd group ($\chi^2 = 9.9$ $p = 0.07$). Closure of decay cavities after 2 months of treatment was observed in 70% of cases in patients of the 1st group, in 53.6% of cases — of the second and in 15.6% of cases — of the third. In 47.4% of patients with G2/G2, destructive changes persisted for more than 4 months of treatment ($\chi^2 = 10.3$, $p = 0.03$).

Patients with genotype G2/G2 have a tendency to a protracted tuberculosis. The study of the polymorphism of the MMP-1 gene in patients with pulmonary tuberculosis can be used as a prognostic criterion of the clinical course of tuberculosis, correction of specific therapy, and justification for prescribing drugs affecting collagen metabolism.

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IN VITRO ACTIVITY OF BEDAQUILINE AGAINST NON-TUBERCULOUS MYCOBACTERIA

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Infections caused by non-tuberculous mycobacteria (NTM) have become more frequent in the last years since they have emerged as important pathogens in immune-compromised patients. Unfortunately, their treatment is long, toxic and often with poor results. Bedaquiline (BDQ) (trade name Sirturo; Janssen Therapeutics, Inc.) has been approved for the treatment of multidrug-resistant tuberculosis and there is increasing interest in its potential use for treating NTM infections.

The aim of this study was to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of BDQ against clinical isolates of NTM obtained from different hospitals. We investigated the possible role of gene mutations on the activity of BDQ against NTM by sequencing the *atpE* gene.

The MIC was determined by the broth dilution method in 7H9 medium supplemented with OADC and glycerol using the resazurin microtiter assay (REMA). The MBC was determined by conventional 7H10 plate agar dilution method.

Range concentration of BDQ in REMA was assessed from 2 to 0.0035 μg/ml. Each experiment was performed in triplicate. The MIC of BDQ was found at ≤ 0.015 μg/ml. The MBC of all NTM tested was found to be higher than 4 times the MIC. No nonsynonymous mutations in the *atpE* gene that conferred BDQ resistance in all NTM tested were identified.

BDQ exhibited a strong inhibitory effect against all the NTM clinical isolates tested. These promising results indicate that BDQ could be potentially useful for the treatment of NTM. However, according to the MBC data it lacks bactericidal activity. Nevertheless, BDQ could still have excellent potential for use in patients with NTM infections and further investigation is needed.

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EMERGING OPPORUNISTIC PATHOGEN *MYCOBACTERIUM ABSCESSUS* IN SLOVENIA: MOLECULAR ANALYSIS OF RESISTANCE GENES COMPARED TO MIC METHOD

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Mycobacterium abscessus complex (MABSC) is a group of three closely related subspecies: *M. abscessus* subsp. *abscessus* (MAA), *M. abscessus* subsp. *bolletii* (MAB) and *M. abscessus* subsp. *massiliense* (MAM). Correct species identification is important, especially for patients with cystric fibrosis (CF), due to distinct molecular resistance profiles among subspecies. Gene *erm(41)* with T/C polymorphism at nucleotide 28 is responsible for inducible macrolide resistance (only in MAA and MAB, in MAM gene is not functional), meanwhile mutation in *rrl* gene causes high-level macrolide resistance. Amninoglycoside resistance occurs with mutation in *rrs* gene.

We aimed to diferentiate Slovenian MABSC isolates to subspecies level and to assess their molecular resistance profile. Selected isolates were further tested with microdilution method to to obtain full phenotypic resistance profile.

Molecular analysis was performed on 37 clinical isolates recovered from 37 patients in the period 2000–2017 from Slovenian National Mycobacterial Collection. GenoType NTM-DR (Hain Lifesciences, Nehren, Germany) was used to identify subspecies and resistance mutations. Antimicrobial susceptibility testing (AST) was performed on selected isolates with reference microdilution method using Sensititre RAPMYCO microplates (TREK Diagnostic Systems, Cleveland, Ohio, USA). Susceptibility and resistance were assessed according to CLSI guidelines.

GenoType NTM-DR showed the highest prevalence rate for MAA 72.9% (27/37), followed by MAB 16.2% (6/37) and MAM 10.8% (4/37). Resistance profile showed that T28 polymorphism in *erm(41)* gene is present in all MAB and MAM isolates and in 88.8% (24/27) MAA iso-

lates. Mutations in *rrl* and *rrs* genes were not detected. AST for 4 MAM isolates confirmed that inducible resistance is not present even with *erm(41)* T28 mutation. AST for 3 MAA with *erm(41)* C28 polymorphism showed MIC values below 2 mg/L which is interpreted by CLSI guidelines as sensitive strain. AST showed that MIC values for amikacin are between 8–16 mg/L interpreted as sensitive and concordant with molecular analysis.

In Slovenia, for macrolide and aminoglycoside resistance, phenotypic and genotypic results of *Mycobacterium abscessus* complex are concordant. Prevalent subspecies is MAA where high percentage of strains have inducible macrolide resistance. No other unknown genetic mutation was present in our isolates that can cause inducible macrolide resistance which is important for treatment patients with CF, where clarithromycin is first drug of choice.

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MOLECULAR FEATURES OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM PATIENTS LIVING IN CLOSED CITY IN THE URAL REGION, RUSSIA

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Novouralsk is a closed town in the Sverdlovsk region, Middle Ural area in Russia, with a total population of 81 500 and travel and residency restrictions. We aimed to identify the molecular-epidemiological features of *M. tuberculosis* circulating in Novouralsk under these specific conditions.

A total of 87 *M. tuberculosis* clinical isolates obtained between 2013 and 2016 from TB patients living in Novouralsk town were analyzed. According to clinical data, 34 (39.1%) of TB patients were HIV-infected. 53 (60.9%) of TB cases were newly diagnosed. Using real-time PCR we divided *M. tuberculosis* clinical isolates into Beijing/non-Beijing genetic groups. Beijing genotype variant B0/W148 was detected by multiplex PCR assay. VNTR loci MIRU26 and QUB26 were used for subtyping Beijing strains. Spoligotyping was used for further subtyping non-Beijing isolates. Drug susceptibility testing for first and second line drugs was performed by absolute consentration method.

Genotyping identified the predominance of the Beijing genotype isolates (75.5%), among new TB cases, that is almost 20% higher than the average for the Ural region ($p < 0.05$). 52.8% isolates belonged to variant Beijing B0/W148. The majority of Beijing isolates — 35 (40.2%) had seven copies in MIRU26 and QUB-26 loci. Nine (10.3%) Beijing B0/W148 isolates had 2 copies in QUB26 locus that was unusual for this genetic cluster; six patients from this group had TB/HIV co-infection. Seven (8.0%) of non-Beijing isolates belonged to SIT35 spoligotype (Ural family). 20.7% of patient had prison history and 72.2% of them were infected with B0/W148 genotype. The MDR prevalence rate was higher than in Sverdlovsk region (66% vs 43.9%, $p < 0.05$) and MDR status was associated with the Beijing B0/W148 genotype (94% and 6% of its isolates were MDR and polyresistant, respectively).

Epidemiological situation with TB in Novouralsk is characterized by high level of TB/HIV co-infection, predominance of Beijing B0/W148 isolates, which is an underlying reason of high level of MDR-TB.

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PREVALENCE AND DIVERSITY OF NONTUBERCULOUS MYCOBACTERIA IN DIFFERENT REGIONS OF THE RUSSIAN FEDERATION

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Today, more attention is being paid worldwide to the nontuberculous mycobacteria (NTM) infections due to their increase in various regions of the world. The prevalence of different NTM species depends on the geographical location. The prevalence and distribution of the NTM species in Russian Federation have not been sufficiently studied to date.

The objective of this study was to demonstrate the diversity of NTM species isolated from patients in different regions of the Russian Federation. NTM were isolated from solid and liquid media from patients with suspected tuberculosis/mycobacteriosis in the period from July, 2013 to June, 2017 and identified using GenoType Mycobacterium CM/AS assay (Hain Lifescience, Germany) and real-time PCR assay co-developed with Syntol LLC (Moscow, Russia). Seventeen NTM species were identified in 1400 cultures from 876 patients. Exact species was identified for isolates from 840/876 (95.89%) patients. The prevalence of slowly growing NTM was 76.5% (643/840). The most common species was *M. avium* (223/846, 25.5%). Also the high incidence rate (in descending order) has been shown for *M.gordonae* (115/846, 13.12%), *M. lentiflavum* (113/846, 12.9%), *M. intracellulare* (80, 9.13%), *M. fortuitum* (83/846, 9.47%), *M. kansasii* (62/846, 7.1%), *M. abscessus* (52/846, 5.9%) and *M. xenopi* (31/846, 3.53%). 16 of 17 identified species were detected in Moscow region. This may be due to a large number of cases analyzed in this region in comparison with other regions. It was shown that NTM species distribution in Central Federal District, European part of Privolzhsky Federal District and Kaliningrad (Northwest Russia) was similar to European countries: MAC 33–39%, *M. gordonae* — 10–20% and *M. fortuitum* — 5–13%. The species distribution of NTM for neighboring Syktyvkar and Perm (North of European Russia) was similar and was characterized by high rates of *M. fortuitum* infection and low rates of *M. avium* infection. NTM, isolated from the border city of Khanty-Mansiysk were also characterized by low occurrence of *M. avium* and prevalence of *M. gordonae.*

To conclude, during the study period, 1400 samples collected from 876 patients in 6 federal districts were analyzed. The greatest species diversity — 16 NTM species — was shown for Moscow region. In most of the regions anayzed, slow-growing NTMs prevailed and the most abundant were species belonging to MAC complex. However, even on a relatively small number of observations, some regional features can be observed.

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MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM BOVIS* ISOLATES FROM CATTLE IN BULGARIA

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The observation of the population dynamics, diversity of genotypes and dissemination of *Mycobacterium bovis* strains in Bulgaria and neighboring countries play

an important role in the modern epidemiological investigations of tuberculosis in animals at the regional and international level. Bovine tuberculosis represents a significant economic burden to the agriculture of the affected countries. From 2000 to 2015 the disease shows cyclicity in private farms in different regions of Bulgaria. This study is a first molecular investigation of animal tuberculosis in the veterinary medicine in country. The macroscopic and microscopic observation of 35 diagnostic materials from slaughtered cattle, received in the National Reference Laboratory of animal tuberculosis were studied with the three molecular methods: RD4-PCR, spoligotyping and MIRU-VNTR. In 27 of the examined lymph nodes we found specific lesions for bovine tuberculosis. The findings were confirmed bacteriologically and by conventional PCR. To differentiate *M. bovis* from other *M. tuberculosis* complex subtypes, we used primers flanking specific deletion (RD4) in the genome of *M. bovis* and obtained the 446 bp DNA product. The spoligotyping subdivided the strains into 3 spoligotypes shared by two to 20 strains. Further molecular investigations of *M. bovis* strains are needed to characterize the genetic diversity and population structure of *M. bovis* strains isolated from cattle in Bulgaria. New information will be added to the global database in the field of molecular epidemiology of the prevalence of *M. bovis* strains in the cattle population in Bulgaria, which will allow comparative analysis with data from the Balkan region and Europe.

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INTERNATIONAL VALIDATION OF ANALYSIS PIPELINES FOR WHOLE GENOME SEQUENCING DATA OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

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The aim of this multicenter study was to validate and compare different pipelines used for analysis of the Whole Genome Sequencing (WGS) data of *Mycobacterium tuberculosis* isolates.

All 535 *M. tuberculosis* isolates of culture positive cases in the Netherlands in 2016, were subjected to WGS, in addition to the routine application of VNTR typing. Transmission suggested on basis of identical VNTR profiles of cases in 2016 was further investigated by municipal health services and 41 epi-links were traced. Fastq.gz files of all 535 samples were analysed in four different WGS pipelines to facilitate international comparison: 1) SNP-based method at the RIVM/Bilthoven/The Netherlands; 2) SNP-based method at Oxford University/UK; 3) SNP-based method and 4) cgMLST at Borstel/Germany.

In all pipelines, shorter than 12 SNP distances between the 41 epi-linked cases was observed. One epi-linked pair revealed a higher genetic distance of 27 SNPs in the Bilthoven pipeline, due to poor sequence quality resulting in low coverage. In general, the genetic distances between isolates of the epi-linked cases were smaller in the Oxford and Borstel pipelines (0–3 SNPs), than in the Bilthoven pipeline (1–11 SNPs). All pipelines clustered roughly the same cases, more isolates without identified epi-links were clustered in the Oxford (n = 34) and both Borstel pipelines (n = 32 in the SNP pipeline and n = 39 in the cgMLST) than the Bilthoven pipeline (n = 29).

Also, some cases not clustered by VNTR were clustered by WGS. Patient characteristics revealed that in some of these pairs of cases an epi-link, missed by VNTR typing, was likely.

Several differences were observed among the pipelines with regard to the version of reference genome used, software used for mapping and SNP calling, (repetitive) regions excluded in the analysis, the minimum number of reads to support SNPs, and the minimum allele frequencies. The RIVM pipeline was adapted in the light of these results to function more in line with other international laboratories pipelines, facilitating the comparability of results.

International standardization on all these variables is necessary, and subsequently on the SNP cut-off to be applied to WGS clustering, to allow international-laboratory comparison of WGS data and reliable investigation of cross-border transmission.

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RNA-BASED DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS*

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Multidrug resistant tuberculosis (MDR-TB) is one of the major WHO health concerns. One of the challenges that hampers the effective response to MDR-TB is the long turnaround time of phenotypic Drug Susceptibility Testing (DST) of *Mycobacterium tuberculosis*. To counter this, new fast and sensitive DNA-based methods were successfully introduced over the last years. However, these (a) are based on the knowledge on resistance mutations, (b) do not distinguish living from dead cells, (c) ignore all intrinsic resistance mechanisms, and (d) ignore the influence of compensatory mutations.

We introduce a next-generation diagnostic test based on quantification of drug-specific RNA biomarkers. The basic principle is that a brief antibiotic exposure triggers specific transcriptional responses in susceptible, but not in resistant, microbes within a few hours. This has the advantage that long culture-dependent steps are avoided, yet the resistance phenotype is detected independent of the specific cause of resistance.

First, the global transcriptional response of two *M. tuberculosis* strains to 10 anti-TB drugs was determined using RNAtaq-Seq. A set of highly responsive genes was selected for each drug and RNA-targeting probes were designed.

Next, the RNA-based DST was developed in 96 well format. In short, 200 µl of a positively flagged MGIT™ (BD) culture is spiked with a drug, while a replicate is incubated in absence of the drug. Multiplex mRNA quantification is performed directly on crude cell lysates using a combination of the bead-based MagPix™ (Luminex) and Quantigene™ Plex (Thermo Fisher) technology.

The normalized expression levels are combined to one numeric value which determines the drug susceptibility of the investigated strain.

We successfully developed 8 primary sets of RNA biomarkers for ten 1st-line, 2nd-line and new drugs. Taking isoniazid as proof of principle, we present a biomarker set of 5 responsive genes and 3 normalizing genes, which enables to distinguish susceptible, low- and high resistant TB strains after 6 hours incubation. Next, preliminary results demonstrate that the biomarker sets can successfully discriminate between susceptible and resistance strains for the selected drugs.

We present a robust, RNA-based DST without the need for RNA extraction. The assay was proven to be efficient for isoniazid. With a total of 8 biomarker sets under optimization, the drug resistance profile of up to 14 drugs can be determined.

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POPULATION STRUCTURE OF *MYCOBACTERIUM TUBERCULOSIS* IN RUSSIAN REGIONS BORDERING EU COUNTRIES

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Population structure of *Mycobacterium tuberculosis* in Russia is characterized by predominance of the Beijing genotype strains. A high burden of multidrug-resistant (MDR) tuberculosis in the northwestern Russia may have a negative impact on TB control programs in the neighboring countries of the European Union.

We aimed to assess the population structure of *M. tuberculosis* in northwestern Russian regions (Karelia, Kaliningrad, Murmansk, Pskov) bordering countries of the European Union (Finland, Norway, Estonia, Latvia, Lithuania and Poland).

A total of 304 *M. tuberculosis* isolates (2013–2017) from newly diagnosed TB patients in geographically distant regions of northwestern Russia were studied: Republic of Karelia (n = 78), Pskov (n = 89), Kaliningrad (n = 73) and Murmansk provinces (n = 66). The Beijing genotype family and its particular clusters B0/W148 and 94–32 were detected by analysis of specific markers in *dnaA-dnaN*::IS*6110*, *Rv2664-Rv2665*::IS*6110* and *sigE98*, respectively. Non-Beijing isolates were subjected to spoligotyping followed by comparison to the SITVIT_WEB.

These 4 Russian regions differed to some extent in the prevalence of the Beijing genotype: from to 55.1% in Karelia, 44.9% in Pskov, 63.0% in Kaliningard and 51.5% in Murmansk. Beijing B0/W148-cluster was identified in 17.9; 6.7; 19.2 and 10.6% isolates, respectively. The prevalence of the Beijing 94–32-cluster did not differ: 28.2; 29.2; 28.8 and 30.3%, respectively. In Pskov and Murmansk, most strains were drug susceptible (62.9 and 62.1%), while 17.9% and 25.8% were MDR, respectively. In contrast, MDR strains prevailed in Karelia and Kaliningard — 51.3 and 43.8%, while 41.0 and 36.9% strains were susceptible. In total, for four regions together, 90.2% (37/41) strains of the Beijing B0/W148-cluster were MDR. Beijing 94–32-cluster strains showed much lower rate of MDR — 34.8%, (31/89) ($P < 0.0001$), while 41.6% (37/89) were susceptible. Among non-Beijing *M. tuberculosis* the largest families were T (14.1 and 15.1%) in Karelia and Kaliningard, LAM (23.6%) in Pskov, and Ural (19.7%) in Murmansk.

M. tuberculosis population in the northwestern Russian-EU border provinces is marked by predominance of the major clonal complexes of the Beijing genotype (B0/W148 и 94–32). However, these clones significantly differed in the proportion of MDR. A circulation of the MDR-associated isolates of the Beijing B0/W148-cluster presents the major concern for local and national TB control programs in the Russian regions and likely adverse impact on the neighboring EU countries.

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DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*: FROM PHENOTYPIC MIC-ANALYSIS TO WGS FOR ROUTINE DRUG SUSCEPTIBILITY TESTING

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The phenotypic methods used for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* generally take weeks or months and require culture and manipulation of large numbers of highly infectious bacilli. Drug resistance in *M. tuberculosis* is almost exclusively a consequence of genomic mutations and so molecular determination of resistance offers a rapid, potentially cost effective, and safer alternative.

In 2016, the SRL in Stockholm introduced whole genome sequencing (WGS) for the molecular typing of clinical isolates of M. tuberculosis. At the same time, the WGS analysis made information of various putative drug resistance-related mutations available, which was utilized to complement the routine phenotypic DST. Since 2016, the WGS information on resistance to all clinically relevant drugs is analysed and routinely reported together with the extended phenotypic DST results for all the Swedish multidrug resistant (MDR) isolates.

Retrospectively, we have performed an observational study to compare the WGS data to the phenotypic DST to predict the resistance phenotype from the genetic sequence. The study included 877 clinical TB isolates (only one sample per patient was included) received by the Public Health Agency of Sweden between 01.01.2016 and 31.12.2017. The analysis of the isolates' resistance profiles for the first and second line drugs, obtained from the WGS and Bactec MGIT 960, is ongoing.

Simultaneously, we are evaluating the microbroth dilution technique used for the MIC determination of 150 consecutive (MDR) *M. tuberculosis* together with the corresponding WGS analysis as a part of a collaboration with the CRyPTIC (Comprehensive Resistance Prediction for Tuberculosis: an International Consortium) network. The MIC determinations of drug susceptible and resistant *M. tuberculosis* isolates are necessary in the work to establish correct critical test concentrations for phenotypic DST as well as to identify the minimal, moderate and high confidence mutations. Such coupled analysis will more accurately define clinical drug resistance and may possibly constitute the DST algorithm of choice in an increasing number of laboratories.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN MONGOLIA: SOURCES AND PATHWAYS OF MDR *MYCOBACTERIUM TUBERCULOSIS* STRAINS

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Mongolia is a country with a high burden of tuberculosis (TB). The emergence and spread of multidrug resistance (MDR) TB in Mongolia is associated with problems of early diagnosis and the possibility of cross-border spread of MDR-TB along the Trans-Siberian Railway line from Russia or China. The objective of this study was to reveal sources and pathways of *Mycobacterium tuberculosis* (MTB) strains in Mongolia.

A total of DNAs of MTB from Mongolia (309 strains) were studied. RD 105/207 and 24 loci MIRU-VNTR typing were applied for genotyping and the results were analyzed by MIRU-VNTRplus application. PCR-real time typing was used to identify subtype CC2/W148 by the specific deletion in the *kdpD* gene. Mongolian MIRU-VNTR patterns of MTB were compared with evaluable published Chinese and Russian profiles.

All tested 309 MTB isolates distributed to four lineages: Beijing (228/309 — 73.8%), LAM (33/309 — 10.7%), T (30/309 — 9.7%), H (9/309 — 2.9%), and orphan (9/309 — 2.9%). 21 clusters uniting 187 strains were identified. Out of 228 Beijing strains 165 were clustered and significantly associated with MDR-TB cases from Ulaanbaatar and big railway stations settlements ($p < 0.001$), but MIRU-VNTR profiles from all Mongolian provinces were common.

24-MIRU-VNTR profiles of Mongolian and Russian strains were differed. The three largest clusters consisted of Beijing strains with MLVA MtbC15-9 profiles of 342-32 (58 isolates), 3819-32 (33 isolates) and 1773-32 (33 isolates). Strains with such profiles were found among Russian isolates in single cases in the Irkutsk, Buryat and Zabaikal regions. Subtype CC2/W148, which was primary source of MDR strains in Russia, not found among Beijing strains in Mongolian cohort. Phylogenetic analysis established high genetic close between Mongolian and Chinese profiles of Beijing strains, but Mongolian isolates were grouped in specific cluster.

We determined that there were no significantly transmission of MDR strains from Russia to Mongolia. Our study confirms possibility of cross-border spread of Chinese strains in the past, but now Mongolia has own MDR strains source.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN EASTERN SIBERIA AND FAR EAST

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The highest burdens of tuberculosis in Russia have Asian regions. Beside of social and health care problems this area gained specific geographic spread of *Mycobacterium tuberculosis* (MBT) genotypes and high level of multi drug resistance (MDR). The aim was to carry out comparative evaluation of distribution epidemic MBT strains in Eastern Siberia and Far East of Russia.

We studied 1419 clinical MTB strains collected in Irkutsk region (598), Buryatia (306), Sakha (Yakutya) (351), Zabaykalsky Krai (65) and Primorsky Krai (99). RD 207, 105 181 analysis and 24 loci MIRU-VNTR typing were applied for genotyping and the results were analyzed by MIRU-VNTRplus application. PCR-real time typing was used to identify subtype CC2/W148 by the specific deletion in the *kdpD* gene.

Beijing strains were dominant and varied from 73.6% in the Irkutsk region (Primorsky Krai (72.7%), Zabaykalsky Krai (64.6%), Buryatia (64.4%)) to 43.3% in Sakha (Yakutia). Our study showed that minor Russian genotypes T (13.3%), Haarlem (7.4%) and especially S (13.5%) presented in Sakha (Yakutia) may reflect the MBT population spectrum existing in Russia before Beijing expansion in the 20th century. Yakut's strains of genotype S is unique epidemic group with high level of MDR (77.4% of all S isolates).

As there are in all Russian regions the subtypes CC1 and CC2/W148 were prevailing among Beijing strains. We identified that fifty percent of the Beijing isolates belonged to CC1 group in Irkutsk region, Primorsky Krai and Sakha (Yakutia). Buryatia and Zabaykalsky Krai had unexpectedly low level of CC1, as there were endemic subtype Beijing BL7 (MIT 642) in a quarter of strains from the Beijing collection. The RD 181 deletion absence, the genetic variation of profiles and the presence common clusters of strains of different ethnic groups patients confirm the occurrence of stable circulation of endemic and epidemic BL7 (MIT 642) subtype among the domestic population. The size of subtype CC2/W148 cluster was various from 26.3% in Irkutsk region cohort [Zabaykalsky Krai (24.6%), Primorsky Krai (22.2%), Buryatia (14.0%)] to 13.7% in Sakha (Yakutia). The significant frequency excess of primary MDR was detected only in CC2/W148 subtype vs other epidemic strains of Beijing and nonBeijing genotypes in all areas except Buryatia, where endemic subtype BL7 (MIT 642) shared MDR burden with CC2/W148.

The genotypic structure of the MBT population in the Asian Russia mainly reflects the epidemic expansion features of the Beijing subtypes.

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MOLECULAR DIAGNOSTICS OF TUBERCULOSIS: CLINICAL ASPECTS AND CHALLENGES OF IMPLEMENTATION

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The modern standard of bacteriological diagnostics includes the following set of diagnostic procedures for simultaneous testing of a clinical specimen from a patient suspected of tuberculosis: luminescent microscopy, cultivation on solid media (Finn and Levenstein–Jensen), use of the liquid medium of the BAKTEK 320/960 analyzer, molecular genetic methods in various formats: real-time PCR, GenXpert TB-Rif, Hain test, biochips. The emergence of new technologies does not lead to the rejection of ineffective classical methods. So, in my opinion, the presence of molecular genetic methods in the algorithm of a clinical doctors allows to abandon the microscopy of a clinical material due to low sensitivity and specificity and thereby

optimize costs. However, all the possibilities of molecular genetic methods are not taken into account. So, if the use of GenXpert TB-Rif is absolutely indicated in the case of acute progressive lesions with impaired vital functions in the patient, this technology is less effective with limited diagnostic processes without destruction and inferior to the effectiveness of real-time PCR. The spectrum of detected mutations associated with the development of drug resistance of the causative agent is not taken into account. If more sensitive and cost-effective PCR-based real-time test systems allow detecting only 8 mutations in three genes (*katG*, *inhA*, *rpoB*) that determine the drug resistance of the MBT in 90% of cases, the increase in migration will lead to an increase in cases of tuberculosis with other genetic markers of resistance. In clinical practice, the high risk of falsely positive results from molecular genetic studies in the analysis of material obtained from instrumental or surgical interventions is almost not considered. There is no discussion of the ineffectiveness of the use of molecular tests in the control of chemotherapy at early stages of treatment with a pronounced activity of the process. A clear algorithm is needed for each clinical task.