Current tuberculosis epidemic in the North-western federal region of Russia: drug resistance, molecular epidemiology and risk factor analysis

Anton Baranov

Supervisors:

Gunnar A. Bjune, MD, PhD, professor; University of Oslo, Norway

Andrey O. Mariandyshev, MD, PhD, professor; Northern State Medical University, Russia

Ulf R. Dahle, PhD, senior scientist; Norwegian Institute of Public Health, Norway

This thesis was submitted as a part of the Master of Philosophy Degree in International Community Health

Department of General Practice and Community Medicine

Faculty of Medicine

University of Oslo

May 2007

Dedicated to my wife Victoria

and son Andrey

CONTENT

ACKNOWLEDGEMENTS

I would like to say words of appreciation to my supervisors and all those people who were directly or indirectly involved in my research.

I am very grateful to **professor Andrey O. Mariandyshev** from the Northern State Medical University, Russia, who introduced me into tuberculosis research and was supervising my work overseas. His energy, optimism and wise advises were an enormous source of support for me during the years spent in Oslo.

Ulf R. Dahle, my supervisor from the Norwegian Institute of Public Health, Norway, gave me a lot of knowledge on molecular genetic methods which are in use in contemporary mycobacteriology. His in-depth understanding of the molecular mechanisms and approaches, along with the broad scope of knowledge on all other aspects of tuberculosis research, really fascinated me and helped a lot.

Also I am very much thankful to **professor Gunnar A. Bjune**, University of Oslo, Norway, for an academic supervision of my research. His open-mindedness and research philosophy opened my eyes on many hidden aspects of research and helped me to comprehend the general picture which often stands behind the laboratory results.

Words of appreciation also go to **Turid Mannsåker** who was not introduced as an official supervisor of my research, but, still, her advises on drug susceptibility testing methods and general supervision of my laboratory work at the National Reference Laboratory for Mycobacteria at the Norwegian Institute of Public Health, Norway, were of great help for me.

I want to acknowledge **Olga Toungoussova, Dominique Caugant, and Per Sandven -** they made a pioneer study in the Arkhangelsk oblast 6 years ago and paved the way for the current study. Some laboratory data from 2004, which is included in this manuscript, were collected by them.

Special thanks go to **Nina I. Nizovtseva**, the main tuberculosis coordinator in the Arkhangelsk region for making things run smoothly in Russia and for administrative coordination of the current study.

Also I want to thank the laboratory personnel at the Russian laboratories in all four oblasts that were included in the current study (Arkhangelsk oblast, Murmansk oblast, Republic of Karelia and Republic of Komi) and the laboratory technicians at the National Reference Laboratory for Mycobacteria at the Norwegian Institute of Public Health, Norway, for technical assistance and friendly attitude. Especially, I want to acknowledge **Irina V. Tarasova**, the head of the laboratory and **Oksana M. Mironyuk**, the head of the MDR-TB department at the Arkhangelsk Regional Anti-tuberculosis dispensary. Special thanks go to the laboratory personnel at the Norwegian Institute of Public Health, Norway: **Elisabet Rønnild, Kari Nilsen, Ann-Kristine Øvrevik, Annika Reichman,**

Bente Forsdahl and Marianne Langseth.

And in final, I never could say enough words of appreciation to **my parents, brother Igor, wife Victoria and son Andrey**, who were sharing with me all the ups and downs of the current study and were supporting all my choices, whatever they were. They are the most important ‘finding’ which I got in this life.

ABSTRACT

Title of the study: Current tuberculosis epidemic in the North-western federal region of Russia: drug resistance, molecular epidemiology and risk factor analysis

Researcher: Anton Baranov

Supervisors: Gunnar A Bjune, MD, PhD, professor (University of Oslo, Norway); Andrey O. Mariandyshev, MD, PhD, professor (Northern State Medical University, Russia); Ulf R. Dahle, PhD, senior scientist (Norwegian Institute of Public Health, Norway)

Settings: The study was carried out in 4 administrative territories of the North-western federal region of Russia Federation. Namely, in Arkhangelsk oblast, Murmansk oblast, Republic of Karelia, and Republic of Komi.

Study design: An epidemiological cross-sectional study. 176 *M. tuberculosis* strains were isolated from tuberculosis patients diagnosed with new cases of pulmonary tuberculosis in the North-western federal region of Russia during 2004, 2005 and 2006. The isolates were tested for drug susceptibility with BACTEC 460 and typed with Restriction Fragment Length Polymorphism (RFLP) method and spoligotyping.

Objectives: *Superior objectives:*

1. To describe the level of drug resistance and the genetic diversity of *Mycobacterium tuberculosis* (*M. tuberculosis*) in the North-western federal region of Russia in new spear-positive civilian patients.

2. To disclose risk factors which are associated with drug resistant strains of *M. tuberculosis* circulating in the North-western federal region of Russia.

Specific objectives:

1. To describe the drug resistance patterns of *M. tuberculosis* isolates to the first-line and some of the second-line drugs in new smear-positive cases in the North-western federal region of Russia to outline the magnitude of the problem in these communities.

2. To describe the genetic diversity of the *M. tuberculosis* population isolated from patients residing in the study area by use of RFLP and spoligotyping.

3. To reveal social, demographical and medical characteristics of the study population that are significantly associated with possession of drug resistant tuberculosis among the current collection of *M. tuberculosis* strain*s* from the North-western federal region of Russia.

Results: Rates of drug resistance for the first-line anti-tuberculosis drugs in *M. tuberculosis* isolates were quite high – drug resistance to isoniazid and rifampicin, the most effective anti-tuberculosis drugs, was detected in 44.9% and 31.3% of cases, respectively. Drug resistance for the second-line anti-tuberculosis drugs was lower, with the highest rates of resistance found to

ethionamid– 32.9% of cases, capreomycin – in 17.0% of all isolates and kanamycin – 14.2%.

By use of spoligotyping we could reveal that there are three main lineages of *M. tuberculosis* which are prevalent in the study area. The Beijing lineage was identified in 46.8% of isolates. 25.1% of all the strains belonged to the T lineage, and 12.2% - to the Haarlem lineage.

By assimilating spoligotyping with RFLP data, we found 5 clades of *M. tuberculosis* isolates, with the biggest clade (about half of all cases) characterized by similar RFLP patterns. This clade mainly contained isolates belonging to the Beijing lineage.

On the other hand, the Beijing lineage, despite the fact of having similar RFLP pattern in most isolates, had one subgroup with heterogeneous RFLP patterns.

The Beijing lineage was found to be significantly associated with multi-drug resistance (MDR), drug resistance to all first-line anti-tuberculosis drugs and drug resistance to ethionamid, in comparison with the other two prevalent lineages of *M. tuberculosis* – T and Haarlem.

Conclusions: *M. tuberculosis* strains circulating in the North-western federal region of Russia possess high rates of drug resistance to the first-line anti-tuberculosis drugs, which have increased in comparison with the collection of strains isolated in Arkhangelsk oblast 6 years ago (1). The core of the current *M. tuberculosis* population was constituted by the Beijing lineage, to which about half of *M. tuberculosis* strains were assigned.

The Beijing family had significantly higher percentage of MDR, drug resistance to all first-line drugs, and drug resistance to ethionamid, than the other two most prevalent families – T and Haarlem. This could be one of the factors favoring the abundance of this particular lineage.

Many RFLP clusters comprised of isolates from different lineages were shared by strains from different territories in the North-western federal region of Russia. This suggested that in order to handle the tuberculosis epidemic in the North-western federal region of Russia, one may need to strengthen anti-tuberculosis programs in all regions.

Reporting of the results: The results of the current study were reported to the population studied, to the local health and community leaders, to funding agencies that supported the study. Some data from the current research was presented in a poster at the 17th European Congress of Clinical Microbiology and Infectious Diseases/25th International Congress of Chemotherapy (31 March-3 April 2007, Munich, Germany) and published as an on-line supplement to the following journals - Clinical Microbiology and Infection, Volume 13, supplement no.1 and International Journal of Antimicrobial Agents, March 2007, Volume 29, supplement 2. One article has been published in a peer-reviewed Russian journal indexed in MedLine (2). Several papers based on the results of the current study will be submitted to peer-reviewed international journals.

LIST OF ABBREVIATIONS

AFB – acid-fast bacilli

ARTD – Arkhangelsk Regional Anti-tuberculosis Dispensary

bp – base pairs

CDC - United States Centers for Disease Control and Prevention

CRISP - clustered regularly interspaced short palindromic repeats

DNA – deoxyribonucleic acid

DR – direct repeats

DST – drug susceptibility testing

DVR - direct variant repeat

EQA - external quality assurance

HIV – human immunodeficiency virus

IS*6110* – insertion sequence 6110

IUATLD – International Union Against Tuberculosis and Lund Diseases

M. tuberculosis – Mycobacterium tuberculosis

MDR – multi-drug resistance

MDR-TB – multi-drug resistant tuberculosis

MIRU – mycobacterial interspersed repetitive units

NIPH – Norwegian Institute of Public Health

OR – odds ratio

PCR – polymerase chain reaction

RFLP – restriction fragment length polymorphism

RNA – ribonucleic acid

SPIDR region - spacer interspersed direct repeats region

WHO – World Health Organization

XDR – extensive drug resistance

XDR-TB – extensively drug-resistant tuberculosis

ZN method – Ziehl-Neelsen method

INTRODUCTION

Tuberculosis has claimed its victims throughout much of known human history. This is an ancient disease that spread in epidemic form among susceptible people. It has had a large economic impact on societies, along with a huge influence on human creativity; on the lives of individuals in music, art and literature (3). One of the biggest recent impacts is noted in the advances in biomedical science and healthcare.

Some estimates suggest that the genus *Mycobacterium* originated 150 millions years ago (4). On the basis of molecular genetic analysis of *M. tuberculosis*, Gutierrez at al. concluded that early progenitor of *M. tuberculosis* was present in the East Africa as early as 3 million years ago and may have infected early hominids at that time (5). However, it is likely that modern strains of *M. tuberculosis*, originate from a common ancestor that underwent an evolutionary bottleneck 15,000-20,000 years ago (4).

During the last few decades, a chain of scientific evidences has improved our understanding of this infection. One of the most dramatic events happened on March 24, 1882, when Hermann Heinrich Robert Koch made his famous presentation *Die Aetiologie der Tuberculose*, to the Berlin Physiological Society (4). In that presentation he demonstrated the identity on the causative agent of tuberculosis – the tubercle bacillus. In 1905 Koch was awarded Nobel Prize in Medicine or Physiology for this elucidation of the etiology of tuberculosis.

Another major event in tuberculosis history which should be particularly noted is the discovery of chemotherapy. Before introduction of modern chemotherapy, about 50% of tuberculosis patients died within 5 years (6). The first discovered therapeutic agent with efficacy in the treatment of tuberculosis was para-amino salicylic acid (PAS) by Jorgen Lehmann in 1943. In 1943 Gerhard Domagh discovered thiosemicarbazone and in 1944 Albert Schatz , Elisabet Bugie, and Selman Waksman reported the discovery of streptomycin, the first antibiotic effective against *M. tuberculosis* (4). For this discovery Selman Abraham Waksman was awarded the Nobel prize in Medicine or Physiology in 1952. Isoniazid, the first oral mycobactericidal drug and rifampicin followed in 1952 and 1957, respectively, starting a new era of tuberculosis treatment.

In the beginning of the 20th century , while knowledge of tuberculosis advanced with the work of Villemin, Koch, von Pirquet, and others, the disease rates began to decline (4). The explanation for this decline remains elusive. Improved social and living conditions, herd immunity resulting from natural selection of a genetically more resistant population, improved nutritional status and understanding of infectious epidemics, as well as pasteurization, sanatorial isolation of infectious cases etc. are all considered important contributors to this rapid decline. However, none of these measures are fully adequate to explain the decline in tuberculosis rates. Falling case rates were accompanied by

an apparent shift of disease occurrence in older individuals.

In Europe and North America this decline has continued up to the present time with temporal resurgence of the disease in some countries during the periods of World War I and World War II (4). In contrast, nowadays tuberculosis remains a leading cause of death from a single infectious agent in low-income countries.

Despite advances in tuberculosis research, available anti-tuberculosis drugs, well established control programs in many countries, and international attention to this disease, it is estimated that more than 50 million people will die from tuberculosis between 1998 and 2020. Under the current tuberculosis control conditions, this number may even approach 70 million (6).

LITERATURE REVIEW

Current world tuberculosis epidemiology

Tuberculosis is the leading cause of death from a curable infectious disease (7). Overall, one-third of the world's population is currently infected with the tuberculosis bacillus (8). 5-10% of people who are infected with tuberculosis bacilli (but who are not infected with Human Immunodeficiency virus (HIV)) become sick or infectious at some time during their life. People with HIV and tuberculosis infection are much more likely to develop tuberculosis.

In 2004 tuberculosis incidence was estimated to be 8.9 million cases per year and mortality from tuberculosis – 1.7 million people (8). Approximately 80% of the estimated number of all new tuberculosis cases worldwide are arising in 22 high-burden countries (9). They are (in alphabetical order): Afghanistan, Bangladesh, Brazil, Cambodia, China, Congo, Ethiopia, India, Indonesia, Kenya, Mozambique, Myanmar, Nigeria, Pakistan, Philippines, Russian Federation, South Africa, Tanzania, Thailand, Uganda, Viet Nam, Zimbabwe.

In 2004, the per capita tuberculosis incidence was stable or falling in five out of six WHO regions, but growing at 0,6% per year globally. The exception is the African region, where the tuberculosis incidence was still rising, following the spread of HIV (9). In Eastern Europe (mostly, countries of the former Soviet Union), the incidence per capita increased during the 1990s, peaked around 2001, and has since fallen (9).

The high tuberculosis incidences in many countries are believed to represent the tip of the problem regarding eradication of tuberculosis worldwide. Drug resistant forms of tuberculosis are becoming more and more common. International medical experts are worried about the spread of *M. tuberculosis* strains harboring drug resistance to several drugs. One of the most dangerous forms of resistance is multi-drug resistance (MDR) which is defined as a resistance to at least isoniazid and rifampicin, the two most effective anti-tuberculosis drugs (10). Patients having drug resistant forms of tuberculosis require specific combinations of second- and third-line anti-tuberculosis drugs. These drugs are often less effective than the first-line drugs; they produce more side-effects and require longer medication periods. As a result, the cure rates among patients harboring multi-drug resistant isolates range from 6% to 59% (10).

Moreover, in March 2006, the World Health Organization (WHO) and the United States Centers for Disease Control and Prevention (CDC) reported extensively drug-resistant tuberculosis (XDR-TB) as a serious, emerging threat to public health and tuberculosis control (11). XDR-TB is defined as resistance to at least rifampicin and isoniazid (which is the definition of MDR), in addition to any fluoroquinolone, and to at least one of the three injectable drugs used in anti-tuberculosis treatment: capreomycin, kanamycin and amikacin (11). Treatment of XDR-TB is even more

complicated than MDR, because the choice of available drugs effective in such forms of tuberculosis is very limited.

In the 1990s World Health Organization and International Union Against Tuberculosis and Lung Diseases initiated a project "Anti-tuberculosis drug resistance in the world" aiming at mapping drug resistance patterns of *M. tuberculosis* to the first-line drugs in different countries of the world. The latest, third report was released in 2004 and it includes data from 77 settings or countries collected between 1999 and 2002, representing 20% of the global total new smear-positive tuberculosis cases (10). According to WHO/IUATLD data, prevalence of resistance in new cases to at least one anti-tuberculosis drug ranged from 0% (Andorra, Malta, Iceland) to 57,1% in Kazakhstan (median 10,2%). The highest prevalence of MDR in new cases of tuberculosis were noted in Kazakhstan (14,2%), Israel (14,2%) Tomsk oblast – Russian Federation (13,7%), Karakalpakstan – Uzbekistan (13,2%), Estonia (12,2%), Liaoning province – China (10,4%), Lithuania (9,4%), Latvia (9,3%), Henan province – China (7,8%), Ecuador (6,6%). Of note is the fact that there were just two oblasts (out of 89) from Russia that were included in the last report.

Drug resistance in previously treated cases was higher (10). The median prevalence of resistance to at least one drug was 18,4%, with the highest prevalence, 82,1% in Kazakhstan. The highest prevalence of MDR in previously treated cases was reported in Oman (58,3%) and Kazakhstan (56,4%). Among countries of the former Soviet Union the median prevalence of resistance to the four drugs was 30%, compared with a median of 1,3% in all other settings.

In that report by WHO/IUATLD, it has also been noted about a significant increase in prevalence of any resistance and MDR in a number of settings in comparison with data from previous reports, both in new and previously treated cases.

Description of the tuberculosis epidemiology is incomplete without mentioning such reservoirs of tuberculosis infection as correctional facilities. In a study on the epidemiology of tuberculosis in European prisons, A. Aerts and colleagues (12) analyzed data from 22 countries. The median tuberculosis notification rate (the total number of tuberculosis patients in prisons over the cumulative number of prisoners) was 232 per 100 000 inmates (0–17 808). In the countries surveyed, tuberculosis incidence rates in prisons were 8–35 times higher than in the total population. Consequently, despite the fact that the majority (90.9%) of the participating countries reported performing active screening for tuberculosis on entry into prison, with a median detection rate of 393/100 000 (42–2362), the burden of tuberculosis in prisons is quite high.

After being released, prisoners, among whom there could be undetected tuberculosis cases or patients who have not yet completed treatment for tuberculosis, also represent an infectious risk outside the correctional facility. Additionally, correctional facilities are not isolated societies as

inmates have contact with non-incarcerated people such as guardians, visitors and medical staff. Prisons thus represent a considerable infectious reservoir which sustains tuberculosis epidemics worldwide.

Tuberculosis in Russia

Overall data on tuberculosis morbidity and mortality in Russia was covered by the state during the years when Soviet Union was isolated from the rest of the world. It is only in the early 1990s, after dismantlement of Soviet Union, when these data became available to the public.

It is known that during much of the 20th century tuberculosis morbidity and mortality rates in Russia were declining (13). As a result, morbidity rates fell to 34,2 cases per 100 000 population in Russia in 1990 (13).

But, after dismantlement of Soviet Union in 1991 followed by military conflicts in Chechnya and economic crisis in 1998, tuberculosis morbidity and mortality raised again. The reasons for that could have been disruption and breakdown of management systems in administrative and medical areas, general economic destruction, military conflicts, negative changes in the environment, intensive migration, increasing unemployment and homelessness, and loss of financial support for tuberculosis programs (13). In 1999, tuberculosis morbidity and mortality rates constituted 85,2 and 20 cases per 100 000 population, respectively (13). Similar trends were observed in the other countries of the former Soviet Union (14).

At the present, due to the flaws in registration and reporting systems of tuberculosis in some parts of Russia, poor access to diagnostic facilities and, in some settings, lack of standardization of diagnostic laboratory routine techniques, overall data on tuberculosis incidence and mortality is unavailable. But on the basis of the available data, some estimates have been proposed.

According to WHO estimates, in 2004 the tuberculosis incidence in Russia was 115 (all cases/100 000 population/year), prevalence 160 (all cases/100 000 population) and mortality 21 (deaths/100 000 population/year) (9). The prevalence of HIV infection in adult tuberculosis patients (15-49 years) was quite low and constituted 6.8% (9).

Available and reliable data on drug resistance of circulating *M. tuberculosis* strains in different parts of Russia is even more fragmented. There are just several areas where adequate and standardized laboratory drug susceptibility testing (DST) techniques for epidemiological analysis have been applied so far. Currently, data on drug resistance in patients from civil sector is available for Ivanovo oblast, Tomsk oblast, Samara oblast, Orel oblast, St. Petersburg, Arkhangelsk oblast; and for some prison settings, namely, Arkhangelsk prison, Samara prison, Mariinsk prison, Kemerovo prison (14). Thise data is presented in Table 1. In all these settings the proportions of drug resistance and MDR in

previously treated cases were higher than in new cases of tuberculosis. In the areas where there are data available for comparison of drug resistance levels between the civil sector and the prison sector (such as in Arkhangelsk and Samara), higher rates of drug resistance of *M. tuberculosis* were found in the prison sectors.

Table 1. Tuberculosis drug resistance in different parts of Russia (modified table is taken with authors kind permission from publication: Toungoussova OS, Bjune G, Caugant DA. Epidemic of tuberculosis in the former Soviet Union: social and biological reasons. Tuberculosis (Edinb) 2006 Jan;86(1):1-10)

Region	Year	Drug resistance	
		New cases	Treated cases
Ivanovo oblast	2000	MDR[1] 9% Isoniazid 22% Rifampin 16% Ethambutol 10% Streptomycin 18%	MDR 26% Isoniazid 33% Rifampin 43% Ethambutol 30% Streptomycin 46%
Tomsk oblast	2000	MDR 7% Isoniazid 19% Rifampin 8% Ethambutol 7% Streptomycin 25%	MDR 27% Isoniazid 43% Rifampin 31% Ethambutol 21% Streptomycin 53%
Samara oblast	2001	MDR 45% Isoniazid 52% Rifampin 58%	
Orel oblast	1999–2000	Isoniazid 17% Rifampin 4% Ethambutol 3% Streptomycin 33%	Isoniazid 38% Rifampin 25% Ethambutol 25% Streptomycin 50%
St. Petersburg	1990	MDR 18%	
	1999	MDR 36%	MDR 22%
Archangel oblast	1998–1999	MDR 14% Isoniazid 37% Rifampin 14% Ethambutol 23% Streptomycin 40%	MDR 60% Isoniazid 73% Rifampin 60% Ethambutol 60% Streptomycin 67%
Archangel prison	2001	MDR 34% Isoniazid 43% Rifampin 34% Ethambutol 31% Streptomycin 70%	MDR 55% Isoniazid 65% Rifampin 55% Ethambutol 35% Streptomycin 65%
Mariinsk prison	1999	MDR 26%	
Samara prison	2001	Isoniazid 60% Rifampin 80%	
Kemerovo prison	1999	MDR 23% Isoniazid 66%	

[1]MDR, multidrug resistance.

The need for standardization and improvement of the quality of routine DST methods currently being used at Russian laboratories is recognized. This is important point in order to be able to get high-quality DST results which are essential for the selection of appropriate anti-tuberculosis drugs and for the description of the tuberculosis epidemiology. In 2003, an official Order by the Ministry of Health giving thorough instructions on performance of routine DST method which is currently being widely used at Russian laboratories – absolute concentration method - was released (15).

Another step was undertaken in 2005, when the Federal System for External Quality Assurance (EQA) of laboratory assays in Russia was launched in order to raise the quality of routine laboratory assays. One of the components of this system is DST of *M. tuberculosis* isolates. Under this EQA system the panel of 20 WHO *M. tuberculosis* strains with well-characterized DST pattern is distributed annually to every peripheral laboratory performing DST of *M. tuberculosis.* The results from peripheral laboratories are then compared with the actual DST patterns of those strains and decision about accreditation of every particular laboratory is drawn.

The tuberculosis epidemic in the North-west Russia

Tuberculosis remains a burning problem of the healthcare system in the North-western part of Russia. In 2004 according to the official statistics, tuberculosis morbidity in the North-western federal region of Russia comprised 65,0 per 100 000 and mortality from tuberculosis – 14,0 per 100 000 (16). In Arkhangelsk oblast, data from annual report of ARTD from 2004 witnesses that morbidity and mortality from tuberculosis constituted 57,9 and 16,8 per 100 000 in the civil sector. But if prison sector is included, then these figures rise up to 72,3 for morbidity and 18,5 for mortality per 100 000.

Epidemiological studies in the North-western part of Russia on drug resistance and molecular epidemiology of *M .tuberculosis* are scarce and future studies should be welcomed. In the civil and prison sectors in Arkhangelsk region, however, information is available (17;18). The authors of that studies revealed that 25,2% of all strains isolated among patients living outside correctional institutions, had MDR and the proportion of the Beijing strains was 44,5% (17). In the prison sector the proportion of MDR in new cases was 34,5%, 76,3% of both new and previously treated cases of tuberculosis were caused by Beijing strains (18).

Data from Arkhangelsk suggests that this region suffers a high incidence of tuberculosis, accompanied by high rates of drug resistance. This poses challenges for the local anti-tuberculosis program and demand special approaches in order to control the tuberculosis epidemic. This may also be the case for other administrative areas within the North-western federal region of Russia. There appears to be a great need to gather epidemiological data in the adjacent regions, in order to get an overview of the current situation related to the spread of drug resistant tuberculosis and different

genotypes of *M. tuberculosis*. Such data may serve as a supplement for decision-makers and medical officials when planning interventions into the current tuberculosis epidemic.

Microbiological testing methods of *M. tuberculosis:*

1. **Microscopy**

Simple microscopy of clinical specimens stained by the Ziehl-Neelsen (ZN) method is one of the most frequently used laboratory technique for detection of *M. tuberculosis*. In low-income countries, ZN staining followed by simple microscopy is widely used because of its low cost, and high specificity and sensitivity. Both specificity and sensitivity are dependant on the procedural standards (19). This method confirms diagnosis of tuberculosis by revealing specific AFB and helps to follow-up the treatment process of each patient. This technique is quite simple and inexpensive. The results of the test are available within 24 hours of sputum collection.

There are some other microscopy methods, which differ in the staining procedures. Kinyoun's cold staining is a modification of the classical ZN method that excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin. The quality of the results obtained through this method were found to be inferior to ZN method (19).

Other methods are auramine and auramine-rhodamine staining followed by flourochrome microscopy. Because it is easier to detect a fluorescent rod against a dark background, the fluorochrome staining method allows the examiner to scan the slide at a lower magnification and thus observe a larger area than with ZN-stained smears. These factors reduce the time for screening and lead to greater sensitivity. The proficiency testing study of 167 laboratories in the United States found that the sensitivity and specificity of the ZN and fluorochrome methods are comparable if the procedural standards are followed (19). The authors concluded that because of the little time required for the fluorochrome method, laboratories with large specimen numbers may prefer this technique. In other cases, the ZN method may be recommended.

2. **Identification tests**

Identification tests are performed to make sure that the bacilli belong to the species within the *M. tuberculosis* complex. Most identification tests are either based on biochemical features of *M. tuberculosis* (for instance, salicylic test, nitrate reduction test, niacin accumulation test) or on genetic analysis (for example, AccuProbe testing kit). Different laboratories chose those test that are affordable, reliable and reproducible.

Traditionally, in Russia, identification of *M. tuberculosis* is done according to the Order by Ministry of Health №109 from 2003 (15). Bacilli are identified by visual inspection of colonies, morphology of colonies, speed of growth, temperature for growth, color of the colonies and salicylic

test.

At the NIPH identification is performed by visual inspection of the colonies, a 16S rRNA gene hybridization technique (AccuProbe; GenProbe Inc., San Diego, Calif.) as well as standard microbiological tests (niacin accumulation and nitrate reduction tests).

3. Drug susceptibility testing methods (including molecular basis for drug resistance)

The knowledge on individual DST patterns of *M. tuberculosis* strains isolated from particular patients is essential for prescription of a relevant and effective drug regimen. It is known that drug resistance in *M. tuberculosis* emerge as a result of mutations in particular areas of the genome, encoding different proteins involved in the metabolism of this microorganism. The pressure by irrelevant chemotherapy favors selection of drug resistant strains possessing such mutations and, in turn, this induces higher rates of primary drug resistance.

All DST methods which are currently available for testing of *M. tuberculosis* isolates could be divided into two groups.

The first group includes *in vitro* phenotypic methods. Those methods utilize the principle of inhibition of growth of *M. tuberculosis* isolates in the presence of anti-tuberculosis drugs in the medium. Many modifications of DST methods based on this approach have been developed. Some of them are based on solid media – absolute concentration, proportion methods etc.; the others are based on liquid media – BACTEC 460, BACTEC MGIT, BacT/Alert etc. Growth detection on liquid media vary: it is resulting release of radioactive carbon dioxide, change of the colorimetric properties of the medium or detection of emerging fluorescence.

In vitro phenotypic methods usually are rather time-consuming because of slow growth of *M. tuberculosis* on media. Nevertheless, most of them are considered to be "gold standards" in DST.

The second group comprised by molecular biology methods which detect mutations in the genome of *M. tuberculosis,* known to confer drug resistance to some specific drugs. Such methods are fast, but require knowledge on the mechanisms of drug resistance to different drugs. At the moment, mutations conferring resistance to rifampicin, isoniazid, ethambutol and streptomycin are most well-characterized

Among rifampicin-resistant isolates mutations within a 81-bp region which is a part of the *rpoB* gene that encode a beta subunit of the DNA-dependant RNA polymerase is common. Mutations in some codons within this core region are more frequently observed among rifampicin resistant isolates. Codons 531, 526 and 516 are associated with high levels of resistance (20).

For isoniazid the situation is more complex – mutations conferring resistance have been reported to occur in four different genetic loci: *katG*, encoding catalase-peroxidase; *inhA* involved in fatty acid elongation; *ahpC* encoding alkyl hydroperoxide reductase, and *oxyR* involved in the

regulation of oxidative stress. In addition, it is believed that mutations in other undescribed genes may also encode isoniazid resistance. Despite this complexity, mutations within codon *315* of the *katG* are reported frequently and are often associated with resistance to isoniazid.

Mutations causing resistance to ethambutol have been found in the *embCAB* which codes for different arabinosyl transferases which have a role in lipoarabinomannan and arabinogalactan synthesis.

Resistance against streptomycin is induced by mutations within *rrs*, which codes for 16SRNA and *rpsL*, which produces the ribosomal protein 12S.

Traditionally, in Russia DST is carried out by absolute concentration method on solid media. This method has several disadvantages. The most important of them is that this method demands quite much time before conclusions about susceptibility or resistance of testing strains can be drawn. Usually it takes 10-12 weeks until final results are obtained.

BACTEC 460 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) is a method which is based on DST on liquid medium (21). The principle of BACTEC 460 method is radiometric detection of the metabolic activity of *M. tuberculosis*. The resulting release of $^{14}C0_2$ from liquid medium is measured automatically using an ionization chamber. The amount of growth, indicated by changes in the growth index (ΔGI) in the medium with known drug concentration as compared to that in the control bottle, is correlated to a presence or absence of resistance in 1% of inoculum. If an isolate grows beyond a specific growth index compared with the control, it is considered resistant to that specific antimicrobial agent (21).

BACTEC 460 is regarded as fast and handy method for routine use (21-23). But it also has several limitations which are well known and include manual loading, potential risk of cross contamination related to invasive reading, lack of computerized data management and accumulation of radioactive waste (21). Despite of these disadvantages, BACTEC 460 has much more advantages. For instance, the results of DST with this method could be available within one week. This method does not have such disadvantages, which are present when performing DST on solid media, as inactivation of antimicrobial agents during preparation of agar and destruction of the drugs during long incubation (24). Moreover, higher reproducibility of this method, i.e. absence of significant discrepancies between laboratories when performing DST for the same strains of *M. tuberculosis*, have been demonstrated (23). And the most important point is that all first-line drugs (21;25) and some of the second line drugs (21;23) have internationally standardized recommendations for usage in this method. That is why, currently, this method is treated as “gold standard” in DST.

4. Molecular typing methods

By use of DNA-DNA hybridization studies it has been demonstrated that the members of the *M. tuberculosis* complex are extremely conserved and 99,9% of their DNA is homologus (26). The first molecular tools to characterize circulating strains of *M. tuberculosis* in order to investigate outbreaks of tuberculosis were implemented in laboratories during the 1990s (27). Since that time a lot of different genetic techniques, using different genomic regions as a probe, has been elaborated for in-depth investigation of strains of *M. tuberculosis* (28;29). These markers, with different levels of strain discrimination, include various short repetitive sequences and insertion sequences (IS), of which IS*6110* is the most commonly used for strain typing (26). Tandem repeats such as the polymorphic GC-rich sequence, the major polymorphic tandem repeat, the triplet GTG, various exact tandem repeats, direct repeats (DR) and mycobacterial interspersed repetitive units (MIRU) have also been used for several applications of strain typing. The DNA polymorphism associated with these genetic markers can be visualized by a variety of methods, such as restriction fragment length polymorphism (RFLP) typing, polymerase chain reaction (PCR), DNA hybridization, sequencing, or a combination of these methods (26).

The choice between DNA typing methods strongly depends on several considerations: they should preferably be reproducible, rapid, inexpensive, easy to perform and directly applicable to clinical material. The degree of discrimination of the method and stability of the marker should be appropriate for the research question to be addressed.

The most commonly used techniques are RFLP typing and PCR-based method – spoligotyping, investigating the direct repeat (DR) genomic region of *M. tuberculosis* (29). These techniques allow one to distinguish between isolates of *M. tuberculosis*. In different parts of the world, including Russia, Beijing family of *M. tuberculosis* has been found to be prevalent and also to be significantly associated with drug resistance (17).

Currently, according to the last international recommendations, the Beijing genotype defined as hybridizing to at least three of the nine spacers 35 to 43 and with an absence of hybridization to spacers 1 to 34 by spoligotyping, or carrying 80% or more similarity to 19 reference RFLP patterns (30). This genotype of *M. tuberculosis* was found to be abundant in a previous study from Arkhangelsk, Russia (17;18).

Risk factors for drug resistance of *M. tuberculosis*

Increasing drug resistance in many pathogenic bacteria is a common event in developing countries nowadays (31). The burden of infectious diseases in developing countries is high and availability of resources for modern diagnostics, treatment and patient management is limited. Low-

quality antibacterials and non-compliance to the standard drug prescription regimens are contributing to the problem. For instance, the increase of drug resistance is observed in enteric pathogens (e.g, *Salmonella enterica* serotype *Typhi*, *Salmonella enterica* serotype *Paratyphi*, *Shigella flexneri*, *Shigella dysenteriae* type I, *Vibrio cholerae*), respiratory pathogens (*Streptococcus pneumoniae*, *Mycobacterium tuberculosis*), sexually transmitted infections (*Neisseria gonorrhea*), nosocomial infections (*Staphylococcus aureus,* gram-negative bacteria) (31). These facts give us special concerns in performing DST methods.

M. tuberculosis is a slowly-growing microorganism. Due to this fact, much time is needed between diagnostics of tuberculosis in an individual patient (which in most settings is diagnosed by finding of typical acid-fast rods in sputum samples) and drawing conclusion about drug resistance pattern of a particular *M. tuberculosis* isolate. It can take up to 2 month before the final DST results become available for an individual patient. But in order to get a picture of a "typical patient having drug resistant form of tuberculosis", which will make health workers to become especially focused on diagnostics and treatment of such person even before getting DST results, risk factor analysis has been applied in many settings of the world.

Many studies have been done for revealing factors associated with drug resistance of *M. tuberculosis*. But the primary concern is risk factors for MDR tuberculosis, because, as it has been mentioned before, this form of tuberculosis is especially costly to handle in terms of resources and dangerous from epidemiological point of view.

In a recent systematic review paper, Faustini A. and colleagues analyzed published literature on risk factors for MDR tuberculosis in Europe (32). Twelve European countries were represented in the review: the former USSR, Poland, Hungary and Turkey from eastern Europe; Italy, UK, France, Switzerland, Spain, Portugal, Netherlands and Germany from western Europe.

The pooled risk was 10.23 times higher in previously treated than in new cases of tuberculosis, but wide heterogeneity has been noted between studies. Study design and geographical area were associated with increased MDR tuberculosis risk estimates in previously treated patients: the risk estimates were higher in cohort studies carried out in western Europe (RR 12.63; 95% CI 8.20 to 19.45) than in eastern Europe (RR 8.53; 95% CI 6.57 to 11.06).

The review found that MDR tuberculosis patients were more likely to be foreign born (OR 2.46; 95% CI 1.86 to 3.24), although the strength of the association was much less for previous treatment. There was a clear association observed between MDR tuberculosis and age under 65 years (OR 2.53; 95% CI 1.74 to4.83), but association was weak and more heterogeneous for ages under 45.

Association between male gender and MDR was found significant (OR 1.38; 95% CI 1.16 to 1.65), but MDR-TB patients were more likely to be male in western Europe, where previous treatment

was the most important determinant of MDR-TB. In eastern Europe, where the risk of transmission is greater, male sex was not a risk factor for MDR-TB.

The pooled risk of MDR-TB and positive HIV status was statistically significant (OR 3.52; 95% CI 2.48 to 5.01). Faustini A. and colleagues warned that this could have been affected by a selection bias occurring in favour of HIV infected individuals among cases studied for sensitivity. This hypothesis is supported by the high proportions of HIV patients (32–52%) among those studied for drug sensitivity, which is unlikely to reflect the actual prevalence of HIV among tuberculosis cases.

In Russia, several studies have been done on revealing risk factors associated with MDR tuberculosis. In Ivanovo oblast, homelessness was the only risk factor for drug resistance in new cases of MDR tuberculosis (33). In Tomsk oblast, factors associated with MDR in new cases of tuberculosis were good residence (it was defined as owning your own home, or residing in a cooperative apartment or community apartment) (OR 2.6, 95%CI 1.1–6.0) and treatment default (OR 5.3, 95%CI 2.4–11.6) (34). Female gender, history of previous treatment for tuberculosis and interruption of present treatment were found significant in a dataset consisting of new and previously treated patients in Arkhangelsk oblast (35). In Samara region, overall multivariate analysis in tuberculosis patients from civil and prison sectors showed that being male, having a history of tuberculosis, previous or current treatment for more than 4 weeks and advanced disease with cavitation are highly significant risk factors for single drug resistance and MDR-TB (36). History of incarceration was found to be significant only in Samara oblast.

OBJECTIVES OF THE STUDY

Our study delineates the magnitude of the problem of drug resistance, in particular MDR, and characterize molecular epidemiology of *M. tuberculosis* strains circulating in the North-western federal region of Russia. This study is covering a wider area than previous studies performed in Arkhangelsk.

Superior objectives:

1. To describe the level of drug resistance and the molecular epidemiology of *M. tuberculosis* in the North-western federal region of Russia among new spear-positive patients.

2. To disclose risk factors which are associated with drug resistant strains of *M. tuberculosis* circulating in the North-western federal region of Russia.

Specific objectives:

1. To determine drug resistance of *M. tuberculosis* to the first-line and some second-line drugs in new smear-positive cases in the North-western federal region of Russia, in order to outline the magnitude of the problem in this community.

2. Describe the molecular epidemiology of *M. tuberculosis* isolated from patients from the study area with IS*6110* RFLP and spoligotyping.

3. To describe social, demographical and medical characteristics of the study population that may be associated with drug resistant tuberculosis in the North-western federal region of Russia.

MATERIALS AND METHODS

Study area

The study was carried out in 4 administrative territories of the North-western federal region of Russian Federation. Namely, in Arkhangelsk oblast, Murmansk oblast, Republic of Karelia, Republic of Komi.

According to the last census of enumeration from 2002, the population of the study areas comprised 1 336 539 people in Arkhangelsk oblast, 892 534 in Murmansk oblast, 716 281 in Republic of Karelia and 1 018 674 in Republic of Komi. Total population at risk is 3 964 028 people or 28,4% of all people registered in the North-western federal region of Russia.

Patients and sample size

A sample of tuberculosis patients who were registered as new cases of tuberculosis at one out of four regional anti-tuberculosis dispensaries serving civil population in Arkhangelsk oblast, Murmansk oblast, Republic of Karelia or Republic of Komi (Russia) was collected.

Because of the long-run practical performance of laboratory testing of the strains of *M. tuberculosis*, time-consuming shipment of the material for testing and requirements for the sample size for this study, we have established following inclusion criteria:

1. Patients from civil sector consecutively diagnosed as new cases of pulmonary tuberculosis during one month in 2004, 2005 and 2006.
2. Isolation of all *M. tuberculosis* strains has been performed before initiation of the treatment with anti-tuberculosis drugs.

   Exclusion criteria:

   Tuberculosis patients previously treated with anti-tuberculosis drugs.

   Representativeness of the sample:

All consecutively diagnosed new cases of pulmonary tuberculosis isolated during one month in 2004, 2005 and 2006 were included in the current study.

Mycobacterial strains

In total, 192 *M. tuberculosis* strains were sent to the National Reference Laboratory for Mycobacteria at NIPH from the Russian laboratories. A total of 16 strains were contaminated or could not be re-cultivated in Oslo. These cultures and corresponding patients were excluded from the current study.

The final number of the strains processed at the NIPH was 176. This number constituted 13 strains from Republic of Komi, 33 strains from Arkhangelsk oblast, 17 strains from Murmansk oblast and 18 strains from Republic of Karelia - all isolated during 2004. During 2005 the NIPH received 25 strains from Arkhangelsk oblast and in 2006, 21 strains from the Republic of Karelia, 19 strains from Murmansk oblast and 30 strains from Arkhangelsk oblast completed the current collection.

Drug susceptibility testing method

BACTEC 460

BACTEC 460 is regarded as "gold standard" in performing DST - that is why we used this method to characterize drug resistance patterns of *M. tuberculosis* strains in our sample.

In brief, procedures for this method involve cultivation of *M. tuberculosis* strains on medium, dissolvement in 0,9% NaCl solution, adjustment to appropriate optical density with photometer, inoculation of bottles containing drugs of known concentrations and daily readings of the results until the criteria for termination of the process are archived.

First-line anti-tuberculosis drugs have well-standardized concentrations for use in this method (21;25) which well correspond to *in vivo* concentration of these drugs. Following drug concentrations were used in our study: isoniazid 0.2 mg/ml, rifampicin 2.0 mg/ml, ethambutol 5 mg/ml, streptomycin 6 mg/ml, pyrazinamid 100 mg/ml.

Concentrations of the second-line drugs are much poorer standardized because of lack of large-scale multicenter studies(21;23;37;38). In our study we used following drug concentrations: amikacin 1.0 mg/ml, ofloxacin 2.0 mg/ml, kanamycin 5 mg/ml. The situation with ethionamid and capreomycin is more complex, because before March 2006, the corresponding *in vitro* concentrations for ethionamid were considered to be 1.25 mg/ml and 5 mg/ml (because of different standards). But in March 2006 two publications based on big experimental databases were published by German and British authors, which proposed another concentration – 2.5 mg/ml (37;38). For capreomycin the situation is simpler – before the beginning of 2006, the recommended corresponding *in vitro* concentrations were1.25 mg/ml and 10 mg/ml. But when the before-mentioned papers were issued, just one concentration remained out of two – 1.25 mg/ml. That is why all *M. tuberculosis* strains received in 2004 and 2005 (n=106) were tested with two different concentrations of capreomycin (1.25 mg/ml; 10 mg/ml) and ethionamid (1.25 mg/ml; 5 mg/ml), and strains received in 2006 (n=70) were tested with just single concentrations of capreomycin (1.25 mg/ml) and ethionamid (2.5 mg/ml). DST with amikacin was carried out just on the strains received in 2006 (n=70), because it is just in 2006 when WHO highlighted the significance of this drug for diagnosis of XDR tuberculosis.

Molecular typing methods

RFLP typing

IS*6110* RFLP typing has been internationally standardized with regard to the choice of restriction enzyme, probe and size markers. This has allowed interlaboratory comparisons by entering DNA fingerprints into a computer and processing with special programs (29). This fact, along with the high discriminatory power of the method, explains why it is widely used in different parts of the world. Several international databases of RFLP patterns from a wide geographical area have been established.

RFLP typing is widely used for studying epidemiology and outbreaks of tuberculosis and for revealing possible laboratory-contamination (false positive samples) because of the high stability of IS*6110*. The half-life of IS*6110* RFLP is estimated to be 3-4 years (39).

This method is based on differences in the number of IS*6110* copies, ranging from 0 to about 25, and their chromosomal positions.

In brief, the procedures for this method are following (29): DNA was extracted from bacterial cultures, purified and digested with restriction enzyme *PvuII*. Restriction fragments were separated on an agarose gel and transferred to a DNA binding membrane. DNA was hybridized to the 254-bp internal PCR fragment of IS*6110* and visualized by use of a digoxigenin-dUTP labeling and detection kit (Boehringer).

Spoligotyping

Spoligotyping represent a rapid method which allow the identification and typing of *M. tuberculosis* complex bacteria even directly in clinical samples. But the level of differentiation by spoligotyping is inferior to that of RFLP typing for strains carrying five or more copies of IS*6110*, but higher for strains with less than five copies.

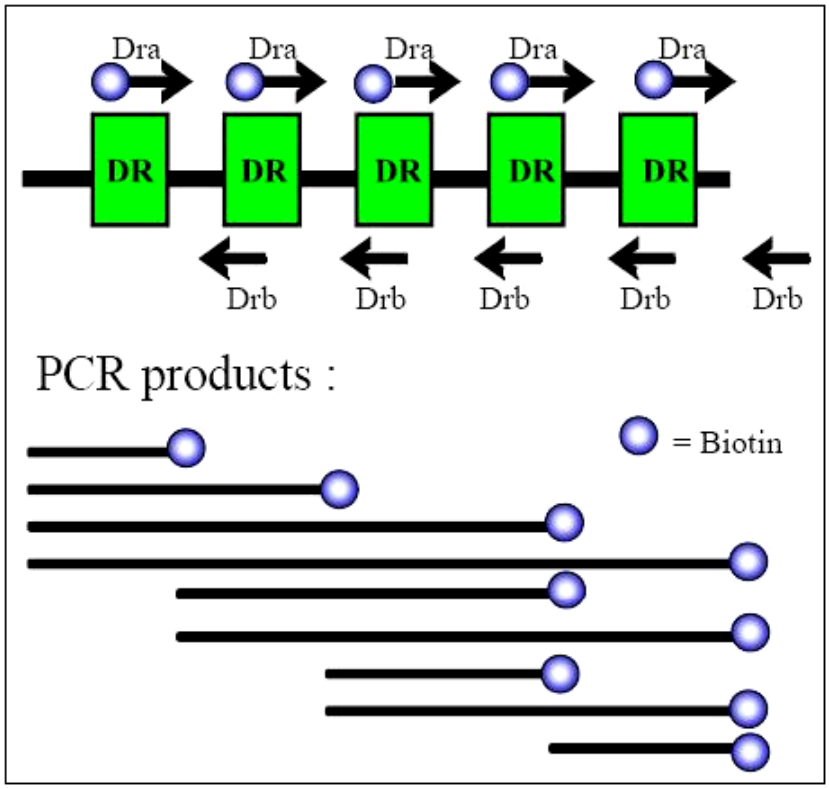
This method is based on polymorphisms present at one particular chromosomal locus, which is called the “direct repeat” region (DR). This region is present in *M. tuberculosis* complex bacteria and was first described by *Hermans et al*, who sequenced this region in *Mycobacterium bovis* BCG (40). This locus has been designated the spacer interspersed DR (SPIDR) region and was recently renamed by Jansen et al as the “clustered regularly interspaced short palindromic repeats” (CRISPR) region (41). This locus contain multiple, well-conserved 36 base pairs (bp) DRs interspersed with nonrepetitive 34- to 41-bp DNA spacer sequences. One DR and its neighbouring non-repetitive spacer is called “direct variant repeat” (DVR). The vast majority of *M. tuberculosis* strains contain one or more IS*6110* elements in the DR region (40).

With spoligotyping, the presence or absence in the CRISPR region of 43 spacers of known sequence can be detected by hybridization of PCR-amplified DNA to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences (40). Differences between

spoligotype patterns have been shown to be due to deletions of spacer sequences in the CRISPR region (40), by transposition of IS*6110* elements (42;43) and, probably, by homologous recombination between adjacent or distant DRs (44).

The first step in the method is to amplify the DR region of a given strain by PCR. The primers used are based on the sequence of the DR, and allow the amplification of the spacer(s) between the DR targets (Figure 1).

Figure 1. Principle of the *in vitro* amplification of DNA within the DR region of *M.tuberculosis* complex bacteria (taken from Spoligotyping kit manual)



The PCR products differ in length because of two reasons: First, the product contains several DVRs; second, the product itself can act as a primer, and become elongated with one or more DVRs. A biotin labeled primer is used, so that all the reverse strands synthesized are biotin labeled. Then amplified DNA is hybridized to a set of spacer oligonucleotides, covalenly linked to a membrane and this membrane is incubated in streptavidin peroxidase, which binds to biotin label on the PCR products. After that, hybridization signals are visualized by enhanced chemiluminescence detection system on X-ray films (40).

The results of the spoligotyping are shown as presence or absence of spots corresponding to

spacers present in the isolate. For the purporse of convenience, these results are transformed into standardized octal code (45).

Spoligotyping is a good method for rapid molecular characterization of *M. tuberculosis* isolates. It helps assign strains into different lineages, and appears well suited for evolutionary studies on the phylogeny of *M. tuberculosis*. This method helps us to get a general picture of the molecular profiles of circulating strains. For individual information related to contact tracing, spoligotyping represents a rough classification tool as it examines a small genomic area of the whole genome. Spacers can only be lost, thus diversity is unidirectional and conclusions of molecular characterization may be difficult to make. However, the significance of this method for molecular characterization and assignment into phylogenetic lineages appear unequalled by other typing methods for *M. tuberculosis* strains.

On the basis of compilation of available data on worldwide spoligotype patterns, the fourth international spoligotype database was published recently (46). It contain data on 39 295 strains from 122 countries, which are tentatively classified into 62 clades/lineages. This is a useful and powerful tool for the identification of *M. tuberculosis* isolates.

Repeated attempts failed to produce reliable spoligopatterns for 5 of the 176 isolates. The troubleshooting to complete these patterns were not considered within the scope of the current thesis. These strains are to be analyzed at a later time.

Data collection

A special structured questionnaire was designed in order to retrospectively collect information from medical records on patient's medical, social or demographic characteristics (see Appendix). We were able to collect information regarding the patients, whose isolates were sent to the NIPH in 2006. This information was analyzed and associations with microbiological characteristics of the individually isolated strains were determined by statistical analysis. Experience from the Arkhangelsk study was taken into account when designing the questionnaire for the current study.

Data analysis

SPSS for Windows, version 14.0, and module 'Statcalc - Statistical calculator' from EpiInfo version 6.04d (CDC, USA and WHO, Geneva, Switzerland) were used for statistical analysis. Associations between proportions were expressed as Odds Ratios. Degrees of association between proportions were assessed using $\chi 2$ with Yate's correction and confidence intervals. If expected values in cells were less then 5, Fisher's exact test was used. Logistic linear regression was applied in search for demographic, social and medical factors which are significantly associated with drug resistance in

M. tuberculosis.

The software "Bionumerics" (AppliedMaths, Kortrijk , Belgium) version 1.5 was applied for the analysis of RFLP patterns of *M. tuberculosis* strains. Before the analysis, RFLP data were normalized according to genetic markers with standardized molecular weight. To analyze the RFLP patterns the Dice similarity coefficient was used with the following position tolerance settings – optimization 1.0%, position tolerance 1.0%, change towards end of fingerprint 0%, minimum height 0%, and minimum surface 0%. Cluster analysis was performed using the unweighted pair-group average method. A cluster was defined as two or more *M. tuberculosis* strains exhibiting 100 percent similarity of RFLP patterns under specified position tolerance settings.

Ethics of the study

The study was conducted according to the Helsinki declaration. All collected information was kept in a safe place without possibility that a third party could access it. In the reports of the results it is impossible to identify identity of any particular tuberculosis patient.

Ethical clearance of the study was evaluated and approved by the Department of International Community Health, University of Oslo, Norway.

Definitions

In our study we used the following official definitions by IUATLD and WHO (47):

A case of tuberculosis: a patient in whom tuberculosis has been bacteriologically confirmed, or has been diagnosed by a clinician.

Pulmonary tuberculosis (sputum smear-positive):

a) two or more initial sputum examinations positive for acid-fast bacilli (AFB), or

b) one sputum smear examination positive for AFB plus radiographic abnormalities consistent with active pulmonary tuberculosis as determined by clinician, or

c) one sputum smear positive for AFB plus sputum culture positive for *M. tuberculosis*

New case of tuberculosis: a patient who have never had treatment for tuberculosis or who has taken anti-tuberculosis drugs for less than one month.

Treatment outcomes for smear-positive pulmonary tuberculosis patients:

a) *cure:* a patient who is sputum smear negative in the last month of treatment and on at least one previous occasion

b) *treatment completed:* a patient who has completed treatment but who does not meet the criteria to be classified as a cure or a failure

c) *treatment failure:* a patient who is sputum smear positive at five month or later during

treatment

d) *died:* a patient who dies for any reason during the course of treatment

e) *defaulter:* a patient whose treatment was interrupted for two consecutive month or more

f) *transfer out:* a patient who has been transferred to another recording and reporting unit and for whom the treatment outcome is not known.

RESULTS

Drug susceptibility patterns of *M. tuberculosis* strains

The results on the prevalence of drug resistance to every single tested first- and second-line drug are presented in Table 2. Strains exhibiting intermediate and resistant results were classified as drug resistant, because we considered intermediate results to be a manifestation of low-level drug resistance. Among the first-line drugs intermediate results were very rare – there were just one strain which produced intermediate result to streptomycin and two strains with intermediate drug resistance pattern to pyrazinamid. Intermediate results for the second-line drugs were obtained for capreomycin at concentration of 1.25 mg/ml – 12 strains, ethionamid at concentration 5 mg/ml – 2 strains and ethionamid at concentration 1.25 mg/ml – 1 strain.

Drug resistant results were obtained in 44.9% of all *M. tuberculosis* isolates when performing DST for isoniazid, 44.3% - for streptomycin, 31.3% - for rifampicin, 30.1% - for ethambutol and 29.0% - for pyrazinamid.

Drug resistance to the second-line drugs was low for amikacin, ofloxacin and capreomycin (at concentration 10 mg/ml) - 4.3%, 3.4% and 1.9% of all isolates, respectively. For capreomycin at concentration 1.25 mg/ml and kanamycin drug resistance was registered in 17.0% and 14.2% of all cases, respectively. The highest rate of drug resistance among second-line drugs was found for ethionamid at concentration 1.25 mg/ml – in 77.4%, then comes ethionamid at concentration 5 mg/ml – 33.0% and ethionamid at concentration 1.25 mg/ml – 32.9% of all cases.

MDR was revealed in 30.7% of all *M. tuberculosis* isolates.

We have found one strain from Arkhangelsk oblast which exhibited XDR pattern, i.e. it was resistant to isoniazid, rifampicin, ofloxacin, kanamycin and capreomycin. In addition it was resistant to the rest of first-line drugs and to ethionamid at concentration 1.25 mg/ml. The only two drugs that it was susceptible to were ethionamid at concentration 5 mg/ml and capreomycin at concentration 10 mg/ml.

Table 2. Results of drug susceptibility testing of *M. tuberculosis* strains isolated from patients in the North-western federal region of Russia with BACTEC 460

	Number of tested isolates	*Number of drug resistant strains, abs.[1](%)*
1st line drugs:		
Isoniazid	176	79 (44.9%)
Rifampicin	176	55 (31.3%)
Ethambutol	176	53 (30.1%)
Streptomycin	176	78 (44.3%)
Pyrazinamid	176	51 (29.0%)
2nd line drugs[2]:		
Kanamycin	176	25 (14.2%)
Ofloxacin	176	6 (3.4%)
Ethionamid (1.25 mg/ml)	106	82 (77.4%)
Ethionamid (2.5 mg/ml)	70	23 (32.9%)
Ethionamid (5 mg/ml)	106	35 (33.0%)
Capreomycin (1.25 mg/ml)	176	30 (17.0%)
Capreomycin (10 mg/ml)	106	2 (1.9%)
Amikacin	70	3 (4.3%)
Selected combinations of drugs		
MDR (isoniazid + rifampicin)	176	54 (30.7%)
XDR (MDR+ofloxacin+one of the following drugs: amikacin, kanamycin or capreomycin)	176[3]	1 (0.6%)

[1] abs. – absolute number of isolates with drug resistance

[2] ethionamid and capreomycin were used in several concentrations because the relevant *in vivo* doses are unknown

[3] In the first two recieved batches of strains, sent in 2004 and 2005 (n=106), drug susceptibility testing for amikacin was not performed. It was performed only for the last batch of strains (n=70), because it is just in 2006 when WHO highlighted the significance of this drug for diagnosis of XDR tuberculosis. As a consequence, rate of XDR strains in our set may be underestimated

Spoligotyping of *M. tuberculosis* strains

To the date of submission of the current thesis we had 171 *M. tuberculosis* strains out of 176 typed by spoligotyping. Spoligotype patterns and lineage assignment based on the SpolDB4 (46) are presented in Table 3.

Based on the spoligotype data, one could conclude that there are three main lineages prevalent in the study area. The Beijing lineage accounted for 46.8% of all isolates, while 25.1% belonged to the T family, and 12.2% were assigned to the Haarlem family. The remaining 15.9% were shared by LAM, MANU, U, CAS and S lineages. Only 3 strains remained undesignated to any known family.

Table 3. Spoligotype patterns of *M. tuberculosis* strains isolated from patients in the North-western federal region of Russia (n=171)

Spoligotype pattern	Lineage[1]	Number of strains (n = 171)	Pooled prevalence of lineages, abs.(%)
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing	76	
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	Beijing-like	1	
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■□□■■■■■	Beijing-like	1	80 (46.8%)
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■□□□	Beijing-like	2	
□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■□□	T1	1	
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■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	10	
■■■■■■□□□□□□□□□□□□■■■■■■■■■■■■■■□□□□■■■□□□□	T1_RUS2	1	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	6	
■■■■■■■■■■■■■■■■■■□■■■□□■■■■■■■■□□□□□□□□□■■	T4	1	
■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	T4	1	
■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	T5_RUS1	5	
■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□□■■■■■■■	T5_RUS1	1	
■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	Haarlem3	2	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	Haarlem3	6	
■■■■■■■□□■■■■■■■■□□□□□□□□□□■□□□■□□□□■■■■■■■	Haarlem4	1	
■■■■■■■□□■■■■■■■■■□■□□□□■■■■□□□■□□□□■■■■■■■	Haarlem4	1	
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■■■■■■■□□■■■■■■■■■■■■■■■□■■□□□■□□□□■■■■■■■	Haarlem4	1	21 (12.2%)
■■■■■■■□□■■■■■■■■■■■■■■■■■■□□□■□□□□■■■□■■■	Haarlem4	2	
■■■■■■■□□■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	Haarlem4	2	
■■■■■■■■■■■□□■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	Haarlem4	1	
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■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	LAM1	1	
■■■■■■■■■■■■■■■■■■□■■■□□□■■■■■■■□□□□■■■■■■■	LAM10	1	
■■■■■■■■■■■■■■□■■■■■□□□□■■■■■■■■□□■■■■■■■■■	LAM9	1	9 (5.3%)
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□□□■■■■■	LAM9	1	
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	LAM9	5	
■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■□□■■■■■■■■■	MANU2	3	
■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	MANU2	1	
■■■■■■■■■■■■■■□□□■□□□□□□□□□□□□□□□□■■■■■■■■■	MANU2	1	9 (5.3%)
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□■■■■■■■■■	MANU2	1	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	MANU2	3	
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■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□■■■■■■■	U	1	4 (2.3%)
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■	U	1	
■■■□□□□■■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■■	CAS	1	1 (0.6%)
■■□■■■■■□□■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	S	1	1 (0.6%)
■□□□□□□□□□■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	Undesignated strain[2]	1	1 (0.6%)
■■□□■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	Undesignated strain	1	1 (0.6%)
■■■■■■■■■■■□□■■■■■■■■■■■■■■■□□□■□□■■■■■■■■■	Undesignated strain	1	1 (0.6%)

[1] Assignment of *M. tuberculosis* strains into different lineages described in SpolDB4

[2] Undesignated strain : Not decribed in SpolDB4

Restriction Fragment Length Polymorphism typing results

The RFLP data of all the strains from the current population are presented in Figure 2.

By use of visual inspection of the RFLP patterns and the dendrogram one may suggest that several clades of *M. tuberculosis* are circulating in the study area.

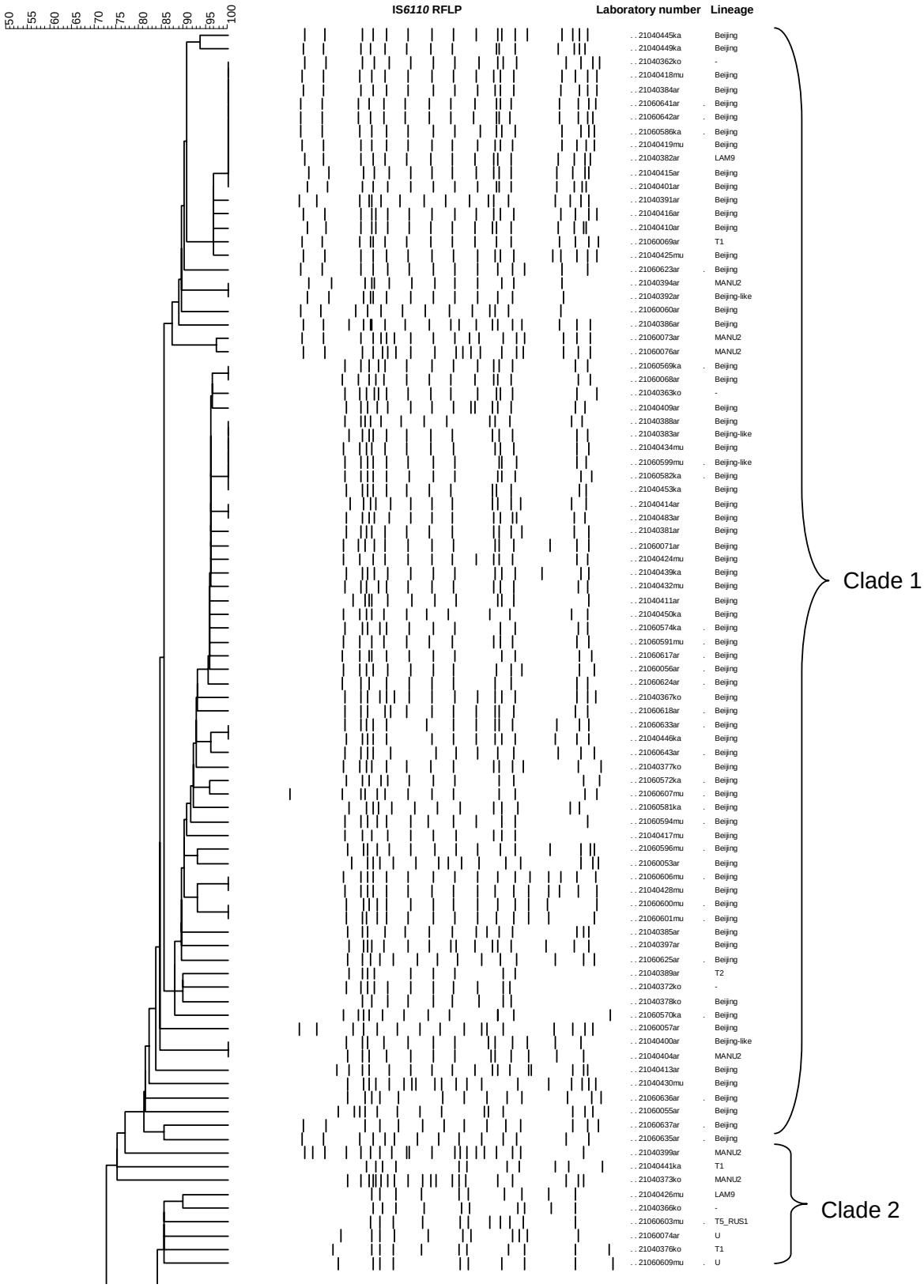
The first clade has a high rate of clustering, which evidence of recent transmission. Most of the isolates assigned to this clade belong to the Beijing lineage. This clade constitute the core of the current tuberculosis epidemic because of high proportion of such isolates (about half of all isolates) and high levels of similarity which suggest recent transmission.

The other 4 clades could be distinguished by combining the spoligotyping data with the RFLP data. These strains do not have the high rates of clustering, as the first clade revealed. However, they are grouped together on the basis of high genetic similarity. The low clustering rates suggest lower transmission rates, but all isolates are closely related to at least one other isolate. This epidemiological characteristic suggests that these strains have been circulating in the area for a long time, and that they represent historic, or indigenous strains. The shallow dendrogram may also suggest that those strains could exhibit longer latency periods, lower virulence or presence of conditions favoring the transmission of first clade strains.

The second clade is formed mainly by the T family. This is the second largest lineage circulating in the community. We found one cluster containing two isolates in this clade and, generally, we can observe that the level of similarity between strains in this clade was higher then in clades 3, 4 and 5.

M. tuberculosis isolates assigned to clades 3, 4 and 5 exhibited highly similar RFLP patterns to other isolates sharing the same clade, but were classified as strains belonging to different families. Most of them belong to T and Haarlem lineages.

Figure 2. Dendrogram demonstrating RFLP patterns of *M. tuberculosis* isolates from the North-western federal region of Russia (n=176)



(continued on the next page)

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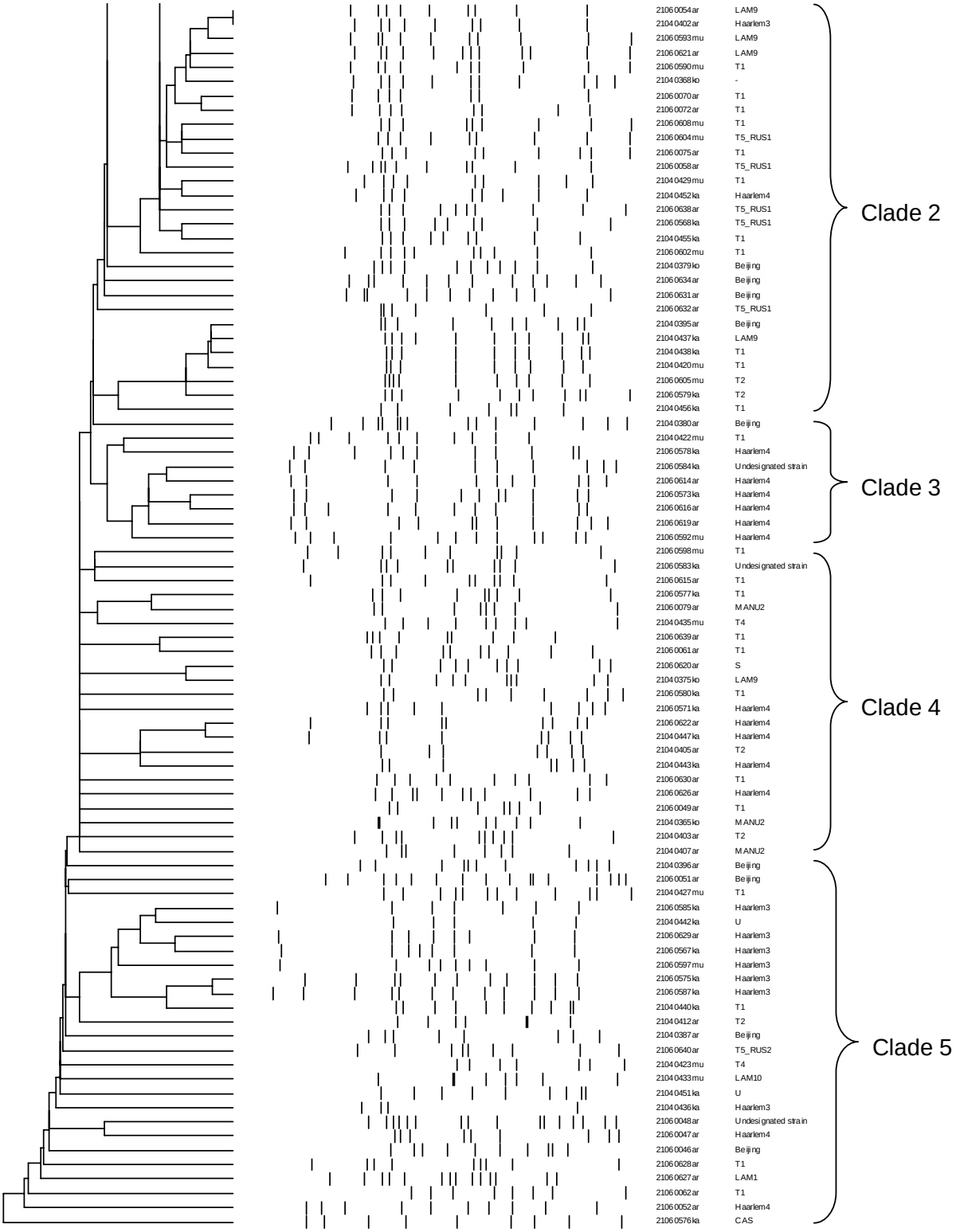


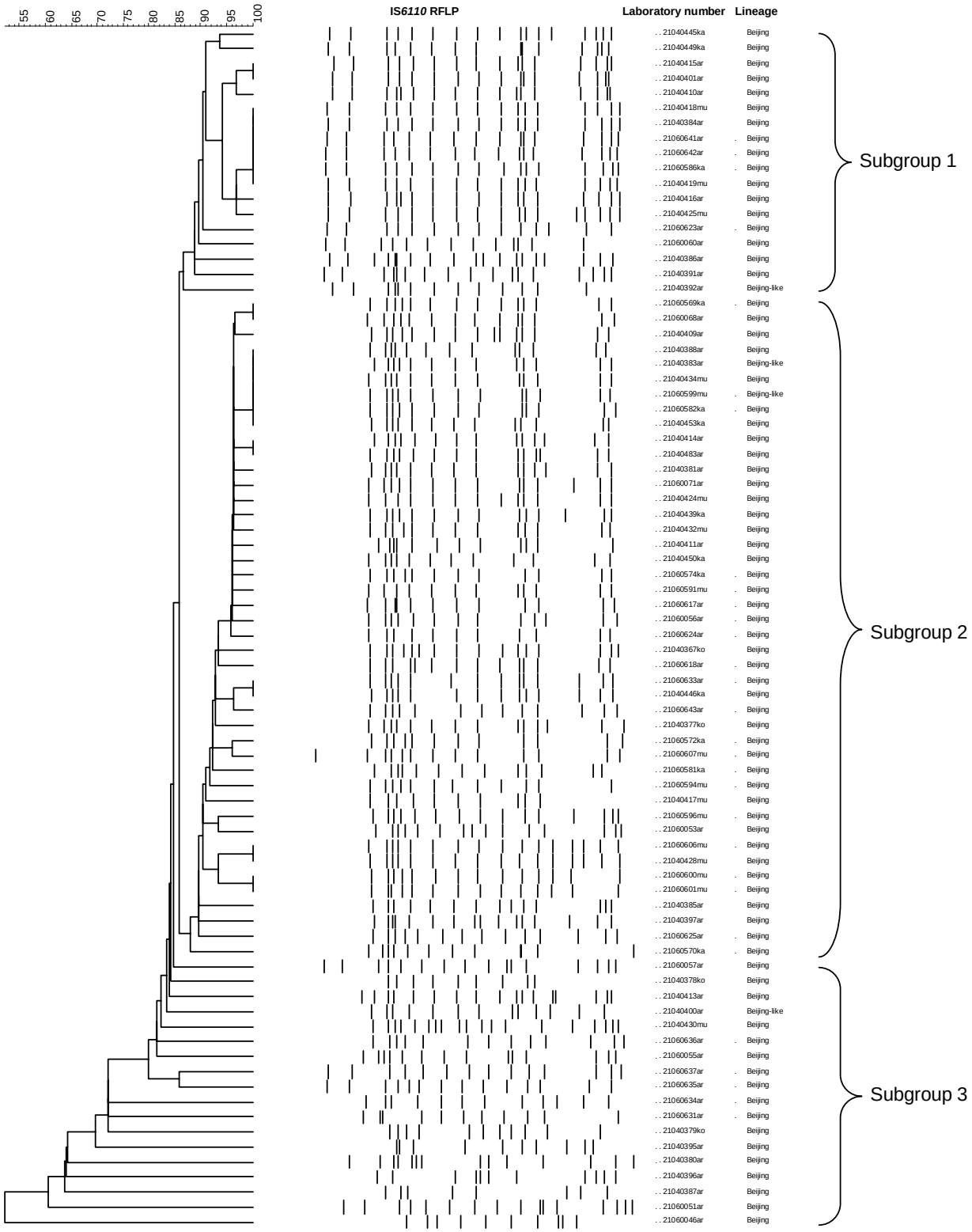
It is well-known that the Beijing strains have a big deletion within the CRISP region, which prevents hybridization with the first 34 spacer oligonucleotides during spoligotyping. This lead to a lower discriminatory power of spoligotyping of the Beijing strains because of the low number of possible variations in spoligotype patterns.

In order to increase the discriminatory power of spoligotyping in Beijing strains, we plotted a separate similarity tree of RFLP patterns of Beijing strains (Figure 3). There we can distinguish 3 subgroups within the current Beijing isolates.

The first subgroup contains 18 isolates (22.5% of all Beijing isolates) and the second subgroup contains 44 isolates (55.0% of all Beijing isolates). These subgroups have high rates of clustering and high similarity between each other. These isolates represent recently transmitted strains in the community.

Third subgroup with 18 isolates (22.5%), on the contrary, is quite heterogeneous in regard to the number and location of IS*6110* sequences. Those strains are not that successfully transmitted, as previous two subgroups. We can speculate that they have lost favoring factor, or they just are varying in some other ways from the first two subgroups.

Figure 3. Dendrogramm built up by RFLP patterns of *M. tuberculosis* strains belonging to Beijing genotype (n=80)



Risk factor analysis

Social, demographic and medical characteristics of the study population are presented in Table 4. Data on patients' education and marital status are not presented because in about half of all analyzed medical records this information was absent.

The mean age of tuberculosis patients was 40.1 years (standard deviation 14.2). Two thirds of all tuberculosis patients were males, most of them were smoking and about half of them were alcohol abusers. Known contact with tuberculosis patients within one year before the onset of the disease was reported just by 20 (40%) patients. Most of the patients were living in flats and half of them were jobless. 8 (16%) patients were previously imprisoned. Chronic obstructive pulmonary disease was found in 5 (10.0%) and diabetes - in 1 (2.0%) patient.

The results of the simple microscopy of the first portion of sputum were reported as negative in 16 (32.0%) of cases (but, still, the bacilli were found later in one or two next sputum portions). 2 (4.0%) sputum samples were classified as 1-9 bacilli in 100 viewing fields. 14 (28.0%) patients produced sputum samples with grade "+". 10 (20.0%) and 8 (16.0%) sputum sample were classified as "++" and "+++", respectively.

We applied logistic regression model in order to find statistically significant explanatory variables among demographic, social and medical characteristics of the patients in regard to MDR-TB and resistance to all first-line anti-tuberculosis drugs. In the analysis we found no statistically significant variables (logistic regression models are not presented in this manuscript). The most feasible explanation for this could be small sample size or actual absence of such explanatory variables.

Table 4. Selected social, demographic and medical characteristics of the tuberculosis patients from the North-western federal region of Russia (n=50)

Variable	Variable value	Number of patients	Percentage
gender	*males*	33	66.0%
	females	17	34.0%
smoking	*no*	17	34.0%
	yes	31	62.0%
	unknown	2	4.0%
alcohol abuse	*no*	26	52.0%
	yes	21	42.0%
	unknown	3	6.0%
known contact with tuberculosis patient within the last one year	*no*	26	52.0%
	yes	20	40.0%
	unknown	4	8.0%
housing	*homeless*	2	4.0%
	hostel	1	2.0%
	flat	42	84.0%
	Own house	5	10.0%
occupation	*jobless*	26	52.0%
	permanent job	17	34.0%
	retired	6	12.0%
	unknown	1	2.0%
previous imprisonment	*no*	40	80.0%
	yes	8	16.0%
	unknown	2	4.0%
chronic obstructive pulmonary disease	*no*	37	74.0%
	yes	5	10.0%
	unknown	8	16.0%
diabetes	*no*	48	96.0%
	yes	1	2.0%
	unknown	1	2.0%

The Beijing genotype was found to be the most prevalent in the study area. Thus, we compared the odds ratios of drug resistance for the Beijing genotype versus other lineages in regard to MDR, resistance to all first-line drugs and separate resistance for each second-line drug (Table 5).

The Beijing lineage was found to possess significantly higher rates of MDR, drug resistance to first-line drugs and drug resistance to ethionamid, when compared to those levels of other prevalent genotypic groups. Resistance to capreomycin, however, was less common among Beijing isolates, than for other genotypes of *M. tuberculosis*. Resistance to this drug was significantly associated with possession of *M. tuberculosis* isolates belonging to the Haarlem genotype.

Table 5. Drug resistance to the first- and second-line anti-tuberculosis drugs in Beijing genotype isolates versus other genotypic lineages of *M. tuberculosis*

	Lineages under comparison				**Odds ratio**	**95% Confidence interval**	**p-value**
	First lineage	*Abs.*[1] *(%)*	*Second lineage*	*Abs. (%)*			
MDR[2]	*Beijing*	*40 (50.0%)*	*T*	*3 (7.0%)*	13.33	3.54-59.01	<0.001
			Haarlem	*2 (9.5%)*	9.50	1.93-63.34	0.002
			others	*8 (29.6%)*	2.38	0.86-6.74	0.11
Drug resistance to all first-line drugs	*Beijing*	*21 (26.3%)*	*T*	*3 (7.0%)*	4.75	1.22-21.49	0.019
			Haarlem	*1 (4.8%)*	7.12	0.91-150.96	0.038
			others	*6 (22.2%*	1.25	0.40-4.0	0.87
Drug resistance to ethionamid (2.5)	*Beijing*	*18 (62.1%)*	*T*	*2 (11.1%)*	13.09	2.17-101.93	0.002
			Haarlem	*1 (6.7%)*	22.91	2.45-534.54	0.001
			others	*2 (25.0%)*	4.91	0.69-43.36	0.11
Drug resistance to capreomycin	*Beijing*	*10 (12.5%)*	*T*	*5 (11.6%)*	1.09	0.31-3.97	0.88
			Haarlem	*8 (38.1%)*	0.23	0.07-0.80	0.01
			others	*7 (25.9%)*	0.41	0.12-1.38	0.13
Drug resistance to kanamycin	*Beijing*	*18 (22.5%)*	*T*	*3 (7.0%)*	3.87	0.98-17.73	0.54
			Haarlem	*0 (0%)*	undefined	undefined	0.02
			others	*4 (14.8%)*	1.67	0.46-6.55	0.56
Drug resistance to ofloxacin	*Beijing*	*5 (6.3%)*	*T*	*0 (0%)*	undefined	undefined	0.16
			Haarlem	*0 (0%)*	undefined	undefined	0.58
			others	*1 (3.7%)*	1.73	0.18-41.06	1.0
Drug resistance to amikacin	*Beijing*	*3 (10.3%)*	*T*	*0 (0%)*	undefined	undefined	0.28
			Haarlem	*0 (0%)*	undefined	undefined	0.54
			others	*0 (0%)*	undefined	undefined	1.0

[1] Abs. – absolute number of strains

[2] MDR – multi-drug resistance (resistance to at least isoniazid and rifampicin)

DISCUSSION

This is the first epidemiological study on the prevalence of drug resistance and molecular epidemiology of *M. tuberculosis* circulating in several areas in the North-western federal region of Russia.

Looking into DST results (Table 2), it was concluded that drug resistance to the first-line drugs was more prevalent than to the second line drugs. This was not unexpected, because first-line drugs have been used for a longer time in the history of treatment of tuberculosis and second-line drugs normally are being used much more rarely.

The fact that drug resistance to the first-line drugs was high, in particular resistance to the most effective anti-tuberculosis drugs – isoniazid and rifampicin (44.9% and 31.3% of cases, respectively), along with a tendency to increase – when compared to the data from a previous study in the Arkhangelsk region (1) - rises serious concerns. In 2001, MDR was diagnosed in 13.5% of new cases of pulmonary tuberculosis. Resistance to isoniazid and rifampicin was revealed in 37.1% and 13.5%, respsectively of all *M. tuberculosis* isolates (1). In the present population resistance to pyrazinamid was found in 29.0% of cases, this poses serious challenges to the treatment regimes of tuberculosis due to the specific pharmacodynamics and pharmacokinetics of pyrazinamid, which make it hard to substitute it with any other drug.

As described in the methodology part, we used capreomycin and ethionamid at different concentrations due to the absence of clear recommendations for drug susceptibility testing of these drugs with BACTEC 460. For ethionamid we used three concentrations and one of them turned out to be too high since 77.4% of all the isolates exhibited resistance at concentration 1.25 mg/ml. The remaining concentrations produced similar results (Table 2). Taking this fact into account, along with the latest recommendations issued by German and British authors (37;38), we assumed that the most relevant concentration for ethionamid is 2.5 mg/ml. In our collection of *M. tuberculosis* strains, drug resistance for ethionamid at this concentration was found in 32.9% of tested isolates.

The various proportions of drug resistant strains to different concentrations of capreomycin, was 15%. In accordance with current recommendations (37;38) we regarded the concentration of 1.25 mg/ml to represent relevant therapeutic doses. The large number of strains exhibiting intermediate susceptibility to this drug, at this concentration may represent emerging low-level drug resistance to capreomycin in the study community. It may also reflect uncertainties related to correct concentrations for capreomycin while performing DST. Standardization of methods for DST to second-line drugs was not considered within the scope of the current thesis, thus the latest internationally validated reference concentration was used. Drug resistance to capreomycin at concentration 1.25 mg/ml was detected in 17.0% of cases.

RFLP represents the gold standard for molecular characterization of *M. tuberculosis* strains. It allows investigation into tuberculosis epidemiology, population diversity, contact tracing and assessment of degree of similarity between different isolates.

We assimilated the data from both spoligotyping and RFLP to describe the dynamics of transmission and prevalence of different *M. tuberculosis* lineages in the current study population (Figure 2). Interpretation of this data suggested the presence of five main clades of *M. tuberculosis*, circulating in the North-western federal region of Russia. The most prevalent clade, contained the Beijing lineage. But this lineage consisted of two similar subgroups as well as a small heterogeneous group of *M. tuberculosis* strains (Figure 3).

Many clusters were found to be shared by strains isolated from patients from different regions in the North-west Russia. It was thus concluded, that despite the distance between patients' residence, they were infected with genetically related strains. This may reflect recent migration processes in North-west Russia, which may pose a challenge to control the epidemic of tuberculosis in isolated areas without simultaneous strengthening of the tuberculosis control programs in adjacent territories.

When looking into the rates of drug resistance to the first- and second-line drugs among Beijing isolates versus other genotypes of *M. tuberculosis* we found significantly higher levels of MDR, drug resistance to first-line drugs and drug resistance to ethionamid, compared to the T and Haarlem lineages.

High rates of drug resistance to first-line drugs among Beijing isolates may represent one factor explaining the high prevalence of this genotype in the study community. Resistant cases may be infectious for longer periods and require longer time for diagnosis. The low efficiency of second-line drugs and possibly altered metabolism of resistant isolates may also promote transmission and resistance to additional drugs. As a consequence, a patient infected with drug-resistant *M. tuberculosis* may remain infectious for a longer period, than a susceptible case. Moreover, in a previous study in Arkhangelsk it was found that some strains from the Beijing lineage did not loose their fitness (measured as speed of growth) on acquisition of drug resistance (48) . This might thus contribute to the success of this particular lineage in the study community.

CONCLUSIONS

1. The core of the current epidemic of tuberculosis in the North-western federal region of Russia is constituted by the Beijing lineage, which was harbored in about half of new cases of tuberculosis. There are also high levels of the T and Haarlem lineages in the region.
2. The Beijing family had the highest clustering levels among the circulating families and constituted two highly similar subgroups as well as one heterogenous subgroup of strains.
3. The Beijing family was significantly associated to MDR, drug resistance to the first-line drugs and drug resistance to ethionamid when compared to the other prevalent families. This may contribute to explain the abundance of this lineage.
4. Many RFLP clusters included strains from different territories in the North-west federal region of Russia.
5. Comparing of the level of drug resistance with the data from previous study in the Arkangelsk region (1), we concluded that there is a trend towards amplification of drug resistance among strains circulating in this geographic area.

RECOMMENDATIONS

We found that many RFLP clusters were shared by strains isolated from patients from different territories in the North-western federal region of Russia, regardless of the clade to which they belonged. This suggested that strengthening of the tuberculosis control programs in all regions are needed, and should be synchronized.

The level of resistance to first-line drugs had increased during the last 6 years (1). Tuberculosis cases with high rates of first-line drug resistance are difficult to cure and require longer diagnostic periods with available DST methods. This prolongs the infectious period for such patients and facilitate resistance to additional drugs. Thus, rapid diagnostics of drug resistance to the first-line drugs, in particular isoniazid and rifampicin, is very important to stop the ongoing transmission of drug-resistant tuberculosis.

APPENDIX

Data collection form

Date of collection of information (дата сбора информации) <____>_____2006

Place where patient was registered (медучреждение, где был зарегистрирован больной)

_____

I. *Patient information (информация о больном)*

1. Culture number in russian laboratory (номер культуры в российской лаборатории) _____

2. Gender (пол): o male (муж), o female (жен)

3. Date of birth (дата рождения) _____ 4. Age (возраст) _____

5. Weight (вес) _____kg (кг)

6. Height (рост)_____cm (см)

7. Marital status (семейное положение):

o married (женат, замужем), o single (холост), o divorced (разведен), o widowed (вдовец) o living together (гражданский брак)

8. Education (образование):

o primary (начальное), o secondary (среднее), o college (среднее специальное),

o incomplete higher (неполное высшее), o higher (высшее).

III. *Medical information (медицинская информация)*

9. Smoking (курение): o no (нет), o yes (да).

10. Narcotics abuse (наркотическая зависимость): o no (нет), o yes (да).

11. Alcohol abuse (алкогольная зависимость): o no(нет), o yes (да).

12. Contact with another patient having tuberculosis (контакт с больным легочным туберкулезом в течение последнего года):

o no (нет), o yes (да)

13. COPD (ХНЗЛ): o no (нет), o yes (да):

13.1 If yes, then (если да, то):

o bronchial asthma (бронх. астма) o chronic bronchitis (хр. бронхит)

o bronchoectatic disease (бронхоэктат. болезнь) o lung emphysema (эмфизема)

o other COPD diseases (хронический облитерирующий бронхиолит, муковисцидоз, биссиноз)

14. Diabetes (диабет): o no (нет), o yes (да).

15. Immunosuppression (иммуносупрессия): o no (нет), o yes (да):

15.1 If yes, then (если да, то):

o oncological pathology (онкопатология)

o intake of hormones and its reason (гормонотерапия, причина)_____

o diseases of connective tissues and autoimmune diseases (заболевания соединительной ткани и аутоиммунные заболевания)

o diseases of blood, specify (заболевания крови, диагноз)_____

o other reason (другая причина) _____

16. HIV status (ВИЧ): o negative (отр), o positive (пол)

If positive, then:

16.1 Has the patient got antiretroviral therapy (проводилось ли лечение антиретровирусными препаратами): o no (нет) o yes (да),

Present TB treatment (настоящее лечение туберкулеза)

17. Diagnosis (диагноз)_____

18. Date of treatment prescription (дата начала лечения) _____

19. Prescribed anti-TB treatment (назначенные противотуберкулезные препараты) _____

_____

Laboratory data (лабораторные данные):

20. Smear microscopy (прямая микроскопия):

If positive, then put the degree (если результат позитивный, то нужно поставить градацию)

20.1 The first smear (первая порция): o neg (отр.) o 1-9 o + o ++ o +++

20.2 The second smear (вторая порция): o neg (отр.) o 1-9 o + o ++ o +++

20.3 The third smear (третья порция): o neg (отр.) o 1-9 o + o ++ o +++

21. Chest X-ray before treatment (рентгенологические данные перед началом лечения):

o normal (без патологии)

o active TB (активный туберкулез)

o TB sequellae (остаточные явления после предыдущего случая ТБ)

o other pulmonary disease, specify (другое легочное заболевание, указать какое)

_____

o unknown (данные или их описание недоступны)

21.1 If active TB on X-ray, describe pattern (если выявлен активный туберкулез, опишите рентгенологическую форму):

o infiltrative (инфильтративный), o cavernous (кавернозный)

o fibrous-cavernous (фиброзно-кавернозный), o miliary (милиарный)

o pleuritis (плеврит)

o atypical (атипичный)

22. Housing (больной проживал): o homeless (бездомный), o hostel (общежитие)

o flat (квартира), o house (дом)

23. Occupation (занятость):

o pupil (учащийся школы или училища), o student (студент), o unemployed (безработный),

o employed (работающий): o physical work (физ. труд),

o mental work (умств. труд),

o medical staff (мед. работник)

o retired (пенсионер)

24. Being in prison (пребывание в заключении): o no (нет), o yes (да)

If yes, then (если да, то):

24.1 How many times (сколько раз)_____,

24.2 Date of the commencement of the last imprisonment and release (месяц и год начала последнего заключения и освобождения): _____, 24.3 _____

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