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Multidrug-resistant *Myobacterium tuberculosis* caused by the Beijing genotype and a specific T1 genotype clone (SIT No. 266) is widely transmitted in Minsk

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ABSTRACT

Setting: This study was performed in the city of Minsk in Belarus, where a very severe problem with MDR-TB was demonstrated in a recent drug resistant survey.
*Objective*The aim of this study was to use molecular typing of MDR and pan-susceptible clinical isolates of *Mycobacterium tuberculosis* to increase the understanding of the transmission patterns and possible differences between the strains causing susceptible and drug-resistant tuberculosis.
Study population and methods: Consecutive isolates from pulmonary TB patients in Minsk were collected at the Belarusian National Reference Laboratory. Isolates found to be either pan-susceptible or MDR were included in the study, which totally comprised 81 MDR and 82 pan-susceptible clinical isolates. All isolates were characterized by spoligotyping. The major clusters were characterized using sequencing of the *pncA* gene.
Results: Three out of four MDR cases were caused by one out of two drug-resistant clones of *M. tuberculosis* belonging to the Beijing and T1 genotypes, respectively. A single T1 clone, SIT No. 266, found exclusively in the MDR cohort, was shown to cause no less than 30% of all MDR-TB cases.
Discussion: The findings indicate that the major cause of MDR-TB in Minsk is an ongoing transmission of certain already resistant M. tuberculosis strains.
Conclusion: The significant transmission of MDR-TB in Minsk underlines the urgent need for strengthened infection control measures to limit the transmission in order to better control MDR-TB.

Introduction

Multidrug resistant tuberculosis (MDR-TB) is a severe threat to effective TB-control as well as to successful treatment of the individual patients. It is estimated by the WHO that globally more than 500,000 TB patients are infected with strains resistant to the most effective anti-TB drugs–rifampicin (RIF) and isoniazid (INH) – and thus classified as MDR-TB cases. Most of these are found in India or China, but it is clearly documented [1] that the highest incidence with up to around 20% of new and 60% of re-treatment cases is to be found in Eastern Europe and especially in some of the countries of the former Soviet Union (USSR).

Belarus, a country in the western part of the former USSR and neighbor of the EU countries Poland, Latvia and Lithuania, is no exception. Even though the reported TB incidence (45/

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100,000) is lower than in its neighboring countries, the high and increasing level of MDR-TB causes severe concerns from a public health perspective and constitutes a very demanding challenge for the national TB-control program. The drug resistance survey (DRS) carried out in the city of Minsk from November 2009 to December 2010 showed the highest ever reported incidence of MDR-TB. Almost 1 out of 2 (48%) of all infectious, smear-positive, pulmonary TB patients was shown to be infected with a MDR-TB strain. Both newly diagnosed and earlier treated patients had an outstanding high MDR incidence, 35.3% and 76.5%, respectively [2]. More recently, a nationwide survey was performed to investigate whether the high MDR incidence was limited to the capital or a more widespread problem. The results showed that the MDR-TB problem in Belarus is widely spread throughout the entire country [3]. It should be understood that this high prevalence of MDR-TB is not a problem limited to a certain city or country. Unpublished information from other parts of the former USSR indicates a broad problem with a high and increasing incidence of MDR-TB in various settings.

It is well documented that certain clones of resistant *Mycobacterium tuberculosis* strains are responsible for a significant proportion of the overall MDR-TB problem in Eastern Europe. Most often these clones belong to the so-called Beijing family of TB strains [4].

This study was the first attempt to increase the understanding of the *M. tuberculosis* strains responsible for the drug resistant TB in Belarus and its transmission. Pulmonary isolates from 81 MDR-TB patients in Minsk city were characterized by molecular strain typing, and the genotypes and level of clustering found were compared with a corresponding number of pan-susceptible clinical isolates.

As a first step, spoligotyping was used to identify clusters and the different geno-families of the studied strains. To further characterize the major spoligo-clusters seen in MDR-TB, the *pncA* gene – the gene where mutations related to resistance to the drug pyrazinamid (PZA) are found – was sequenced. In selected cases, the results were confirmed with MIRU/VNTR. The use of this sequencing for molecular epidemiology of PZA-resistant *M. tuberculosis* strains is an interesting option since the resistance-related point mutations are scattered all over the *pncA* gene with no specific mutations being commonly found [5].

Study population and methods

Consecutive isolates from culture-verified cases of pulmonary TB in Minsk were collected between 2009 and 2010 at the Belarusian National Reference Laboratory (NRL) in Minsk. Isolates found to be either susceptible to all tested first-line drugs (RIF, INH, streptomycin and ethambutol) or MDR (resistant to at least RIF and INH) were included in the study. The study totally comprised 163 clinical isolates from patients with pulmonary TB in Minsk city, 81 isolates being MDR and 82 pan-susceptible.

All strains were isolated at the Belarusian NRL in Minsk on Lowenstein–Jensen (LJ) medium and identified with standard biochemical tests. The *in vitro* susceptibility testing (DST) was carried out with the absolute concentration method on LJ medium with the following critical concentrations: INH 1 mg/L, RIF 40 mg/L, ethambutol 2 mg/L and streptomycin 4 mg/L. DST at the NRL in Minsk was externally quality assured by the WHO Supranational Reference Laboratory at the Swedish Institute for Communicable Disease Control (SMI) in Stockholm.

The molecular characterization of the isolates was carried out at SMI to identify clusters and the different geno-families of the *M. tuberculosis* strains.

All isolates were sub-cultured on LJ medium at SMI. For spoligotyping, mycobacterial lysates were prepared by re-suspending two 10 μl loops of bacteria in 250 μl of 1× TE buffer. After heat-killing the bacteria at 80 °C for one hour, the suspensions were centrifuged at 13,000 rpm for two minutes. The supernatants were discarded and the pellets re-suspended in 500 μl of 150 mM NaCl. This centrifugation and suspension steps were repeated. The final pellet was then dissolved in 25 μl of 1× TE buffer.

Thereafter, all isolates were genotyped with spoligotyping according to the standard protocol [6] using a commercial kit (Isogen Bioscience, BV Maarsen, The Netherlands). Briefly, the DR region of the TB genome was amplified using primers DRa and DRb, and the amplified biotinylated products hybridized to a set of 43 oligonucleotides covalently bound to a membrane. The hybridized PCR products were then incubated with streptavidin–peroxidase conjugate and the membrane exposed to chemo luminescence (Amersham ECL Direct™ Nucleic Acid Labeling and Detection System, GE Healthcare Limited, UK) and exposed to X-ray film (Amersham Hyperfilm™ ECL, GE Healthcare Limited, UK) according to the manufacturer's instruction. The X-ray film was developed using standard photochemical procedures after 20 min exposure. The DNA extracts of the *M. tuberculosis* reference strain H37Rv and of *Mycobacterium bovis* BCG were used as controls.

The patterns obtained were analyzed using Bionumerics software version 5.1 (Applied Maths, Belgium). A cluster was defined as two or more strains sharing identical spoligotyping patterns. Spoligotypes in binary format were converted to an octal code for comparison with the SITVIT2 proprietary database of the Pasteur Institute of Guadeloupe.

The octal codes found were entered in the SITVIT2 database, which is an updated version of the previously released SpolDB4 database [7]. In this database, SIT (Spoligotype International Type) designates spoligotypes shared by two or more patient isolates included in the database. Major phylogenetic clades were assigned according to signatures provided in the database, which defined 62 genetic lineages/sub-lineages. The SITVIT2 contains more than 3000 SITs with global genotyping information on about 74,000 *M. tuberculosis* isolates from over 160 countries of origin.

The *pncA* gene was sequenced in 76 MDR and 78 pan-susceptible isolates as earlier described [5]. Confirmatory MIRU/VNTR analysis was performed in selected strains in the Beijing and T1 clusters of MDR-TB isolates.

Results

Both the proportion of strains in a cluster (28% vs 16%), and the size of the largest clusters (42% vs 30%), were higher