Predominance of multi-drug-resistant LAM and Beijing family strains among Mycobacterium tuberculosis isolates recovered from prison inmates in Tula Region, Russia

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INTRODUCTION

Tuberculosis (TB) is one of the most prevalent infections among adults worldwide. An epidemic has been growing in past decades and the spread of multi-drug-resistant (MDR) forms (i.e. resistant to at least rifampicin and isoniazid) of the disease has been remarkable. Russia is recognized by the World Health Organization as a country with a high incidence of TB. Insufficient funding of the healthcare system, lack of continuous population surveillance and delayed diagnostics, together with the AIDS epidemic, have led to the spread of TB. Each year, more than 20,000 people die from TB in Russia, corresponding to about 80% of infectious disease-related mortality (Shilova, 2003).

TB remains a social disease. Prisons serve as TB reservoirs and thus have a significant impact on the incidence of TB in the civilian population. Several outbreaks of TB have been documented worldwide in communities and hospitals where the source was probably a prison inmate (Portugal et al., 1999; Ikeda et al., 1995). Overcrowding, poor ventilation systems, malnourishment, late case finding and inadequate treatment are common in Russian penitentiaries (Bobrik et al., 2005). As a result, the incidence of TB cases in penitentiaries is substantially higher than that for the entire country (Kononets et al., 2004).

Drug-resistant TB is an emerging global problem and Russia is not an exception. Inadequate treatment is believed to be the primary cause of acquired drug resistance. Many MDR TB cases are identified only when patients fail to respond to conventional therapy, resulting in increased transmission of MDR strains. The spread of MDR TB in prison settings is of major concern. In 2003, 15% of newly registered cases in Russian prisons were MDR (Kononets et al., 2004).

Drug-resistant TB poses a serious burden on the economy. Treatment of MDR TB requires up to 2 years of therapy, involving up to 100-fold more expensive treatment regimes compared with non-resistant TB (Chaulk & Kazandjian, 1998). Early detection of MDR TB cases is a high-priority component of TB control programmes. Phenotypic drug susceptibility testing can take 4–8 weeks (Hacek, 1992; Kent & Kubica, 1985). The need for fast, inexpensive and user-friendly methods for identification of MDR TB is obvious. The application of molecular tools has the potential to decrease the time necessary for detection of drug resistance of Mycobacterium tuberculosis to a few days or even less, thus a patient’s treatment regimen could be adjusted more rapidly and further transmission could be prevented.

Abbreviations: ASD-PCR, allele-specific depletory PCR; ETR, exact tandem repeat; FP, fingerprint pattern; MDR, multi-drug resistant; PGG, principal genetic group; RRDR, rifampicin resistance-determining region; ST, shared type; TB, tuberculosis.

Tables showing sequences of primers used, drug resistance profile and genotypic characteristics of the M. tuberculosis isolates and prevalence of drug resistance according to spoligofamily and case category and a figure showing identification of principal genetic groups using ASD-PCR are available as supplementary material in JMM Online.
We report here the genotypic characteristics and drug susceptibility profiles of clinical isolates of *M. tuberculosis* from a prison hospital in the Tula region (central Russia) during 2001 and 2002. In addition, we assessed an in-house PCR-based assay (Dubiley *et al.*, 2005) for determining the mutations associated with resistance to rifampicin and isoniazid that are predominant in central Russia.

**METHODS**

**Patients.** The study included 87 culture-positive patients with pulmonary TB who were diagnosed and treated in the Ozerki prison hospital (Tula region) between June 2001 and June 2002. A total of 103 patients with pulmonary TB were admitted to the hospital during this period. Specimens were collected once from each of the 103 patients and forwarded to the laboratory after removal of patient identifiers. Sixteen of the collected specimens were considered to be contaminated and were excluded from the analysis.

**Bacterial strains, drug susceptibility testing and molecular characteristics of rifampicin and isoniazid resistance.** *M. tuberculosis* isolates were cultivated on Lowenstein–Jensen medium. *M. tuberculosis* was confirmed using a combination of growth, macroscopic and microscopic appearance and biochemical characterization. The *M. tuberculosis* H37Rv strain was used as a reference for all microbiological and molecular tests.

Drug susceptibility testing was performed by using the absolute concentration assay as described by Kent & Kubica, 1985. Drug concentrations used were as follows: 1 μg isoniazid ml⁻¹, 5 μg streptomycin ml⁻¹, 30 μg kanamycin ml⁻¹ and 20 μg rifampicin ml⁻¹.

The rifampicin resistance-determining region (RRDR) of *rpoB* and codons 300–500 of *katG* were analysed for the presence of mutations by sequencing PCR-amplified fragments of the genes. Reactions were carried out using a CycleReader DNA Sequencing kit (Fermentas), according to the manufacturer’s protocol. rslor and rslrev primers were used for sequencing the segment of *rpoB*, and kslfor and kslrev for the *katG* fragment (primer sequences are available in Supplementary Table S1 in JMM Online).

The allele-specific depletory PCR assay (ASD-PCR) targeting codons 516, 526 and 531 of *rpoB* and codon 315 of *katG* was performed as described previously (Dubiley *et al.*, 2005).

**Molecular epidemiology.** IS6110-RFLP was performed according to the standard protocol as described by van Embden *et al.* (1993). Fragment lengths were determined against an external molecular size marker ranging from 0-7 to 13-1 kb. Fingerprints were scanned and analysed using GelCompar II software (version 2.5; Applied Maths). Patterns were compared by using the unweighted pair group method of arithmetic mean (1% band position tolerance) with Jaccard’s similarity index.

Spoligotyping was performed as described elsewhere (Kamerbeek *et al.*, 1997). The number of tandem repeats at each of the exact tandem repeat (ETR) A to E VNTR loci was determined as described by Frothingham & Meeker-O’Connell (1998). The number of 77 bp repeats was counted in the ETR-D locus.

An in-house ASD-PCR assay targeting polymorphic nucleotides in codon 95 of *gyrA* and codon 463 of *katG* was used for identification of the principal genetic group (PGG; Sreevatsan *et al.*, 1997). Two complementary PCR reactions differing in allele-specific primer sets were carried out for identification of polymorphisms in these positions. The PCR mixtures contained 0-1 μM each katG-Rev and gyrA-Rev primers, 0-2 μM each ISFor and ISRev primers, approximately 10 ng genomic DNA, 40 μM each dNTP and 2 U Taq DNA polymerase (Promega) in the buffer recommended by the manufacturer. In addition to those listed above, the first PCR reaction mixture contained 0-5 μM each R463katG and S95gyrA primers and the complementary one contained the same concentration of each L463katG and T95gyrA allele-specific primers (primer sequences are available in Supplementary Table S1 in JMM Online). Amplification was carried out under the following conditions: initial denaturation at 96 °C for 5 min, then 30–40 cycles with denaturation at 95 °C for 40 s, annealing at 62 °C for 20 s and elongation at 72 °C for 60 s. PCR products were detected by using electrophoresis in ethidium bromide-stained 1% agarose gels (see Supplementary Fig. S1 in JMM Online).

**RESULTS**

**Study population**

The 87 patients included in this study were male, aged between 20 and 56 years (mean age, 35.9 years). All were residents of the Tula region and were incarcerated in the prison facility located in this region; their terms of imprisonment ranged from several months to 14 years. Pulmonary consolidation and fibrocavitary forms of TB were observed in 76 (87.4%) and 8 (9.2%) cases, respectively. None of the patients were HIV-seropositive. Patients were divided into two groups [as recommended by the World Health Organization (1993)]. The first group comprised newly diagnosed sputum smear-positive patients who had not received anti-TB medication previously or had been treated for less than a month. The second group comprised smear-positive patients who had previously taken one or more drugs for more than 1 month. Sixty-three patients had been treated previously and 24 were newly diagnosed cases. No substantial difference in terms of imprisonment was found in these groups. The mean age of newly diagnosed patients was similar to that of previously treated patients (34.2 and 36.7 years, respectively).

**Genotypic classification of strains**

Isolates were assigned to one of three principal genetic groups based on the combinations of polymorphisms at *katG* codon 463 (R > L, CGG > CTG) and *gyrA* codon 95 (S > T, AGC > ACC). Five isolates (5.7%) were found to belong to PGG 3, and 44 (50.6%) and 38 (43.7%) to PGG 2 and 1, respectively. Detailed genotypic characteristics of each strain are presented in Supplementary Table S2 in JMM Online.

IS6110-RFLP analysis of the 87 isolates revealed 45 distinct IS6110 fingerprint patterns (FPs). The number of IS6110 copies per genome varied from 8 to 20, with a mean of 12.56 insertions. Twelve clusters composed of 100% identical IS6110 patterns were identified. VNTR- and spoligotyping results are summarized in Table 1. Spoligopatterns of 87 isolates produced 13 distinct profiles. Spolipatterns were compared with those published in the International Spoligotyping Database of the Instituto Pasteur de Guadeloupe (Bruday *et al.*, 2006). Two isolates (2.3%)...
had unique spoligopatterns not found in the database, whereas 85 (97.7%) patterns were of the shared type (ST). Two major spoligotypes, represented by 35 and 27 isolates, were ST1 (Beijing) and ST252 (LAM09), respectively.

Thirty-eight isolates (43.7%) had spoligopatterns characteristic of the Beijing family (ST1 and 265). Two families, T1_RUS2 and T1, were represented by two (2-3%) and three isolates (3.4%), respectively. Supplementary Table S2 in JMM Online shows that 39 (44.8%) isolates comprised a clearly distinguishable group according to IS6110-RLFP patterns. They shared identical VNTR profiles (22222). All these isolates belonged to PGG 2 (Table 1). This allowed us to recognize ST251, ST266, ST254, ST252, ST42 and isolate no. 741 as members of the LAM family. Similarly, a group of five isolates (ST262, ST1447 and isolate no. 755) was attributed to the Haarlem family.

<table>
<thead>
<tr>
<th>Spoligopattern</th>
<th>No. of isolates</th>
<th>Spoligotype ID*</th>
<th>Family</th>
<th>VNTR code†</th>
<th>PGG</th>
</tr>
</thead>
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<tr>
<td></td>
<td>3</td>
<td>ST265</td>
<td>Beijing</td>
<td>42425</td>
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<tr>
<td></td>
<td>35</td>
<td>ST1</td>
<td>Beijing</td>
<td>42425</td>
<td>1</td>
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<tr>
<td></td>
<td>1</td>
<td>ST251</td>
<td></td>
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<td></td>
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<td>ST266</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Orphan‡a</td>
<td>LAM</td>
<td>22222</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>ST252</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>ST254</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ST42</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1</td>
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<td>42423</td>
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<td></td>
<td>1</td>
<td>ST1447</td>
<td>Haarlem</td>
<td>42522</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>ST262</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>ST33</td>
<td>T1</td>
<td>32423</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ST280</td>
<td>T1_RUS2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Identified according to the International Spoligotyping Database of the Institute Pasteur de Guadeloupe.
†Number of tandem repeats at each of ETR A to E VNTR loci.
‡Spoligotype not present in the International Spoligotyping Database: a, isolate no. 741; b, isolate no. 755.

### Phenotypic and genotypic characterization of drug resistance

Analysis of drug resistance in relation to case category revealed that the highest rate of resistance was to streptomycin, followed by isoniazid and rifampicin for both new and previously treated cases. Results of drug resistance testing are summarized in Table 2 (for detailed data on each strain see Supplementary Table S2 in JMM Online). Overall, the rate of resistance to individual drugs was similar or slightly lower than that reported for Serpukhov prison (Shemyakin et al., 2004). Two isolates (2.3%) were resistant only to streptomycin and two isolates (2.3%) were rifampicin-monoresistant. No monoresistance to isoniazid or kanamycin was observed. Forty-nine isolates (56.3%) were resistant to the four drugs tested. All MDR isolates were resistant to streptomycin. The MDR rate was
37-5% among new cases, which was similar to that reported in Archangelsk (34%) and Samara (37-3%). The MDR rate among previously treated cases was higher (84-1% in Ozerki versus 55% in Archangelsk and 53-6% in Samara) reflecting an even worse situation with TB treatment in the Ozerki prison compared with the other cited penitentiaries in Russia.

PCR and sequencing techniques were used to determine the mutations conferring rifampicin and isoniazid-resistance in the isolates studied. All isolates were subjected to an ASD-PCR assay targeting codons 516, 526 and 531 of \( rpoB \) and codon 315 of \( katG \) and sequencing of the RRDR of \( rpoB \) and a fragment of \( katG \). The ASD-PCR and sequencing data showed that all isoniazid-resistant strains (67/67) carried the \( S > T \) substitution in codon 315 of \( katG \). No other mutations were found in the \( katG \) segment analysed. Results of the mutation analysis in \( rpoB \) are summarized in Table 3. Most rifampicin-resistant isolates in this study (55/66, 83-3%) carried the DS16V or SS31L allele of \( rpoB \). No mutations in RRDR were detected in five rifampicin-resistant isolates. Two of these isolates were rifampicin-monoresistant, one was resistant to both rifampicin and streptomycin, and two were resistant to three drugs, rifampicin, streptomycin and isoniazid. The results obtained by ASD-PCR were in complete agreement with the sequencing data.

### Molecular epidemiology

Fifty-four isolates (62-1%) were members of the 12 clusters identified. The number of isolates per cluster varied from 2 to 17, with a mean of 4-5. Seven clusters consisted of 2 isolates, 3 clusters consisted of 6 or more isolates. No significant difference in the ratio of multi-drug resistance or resistance to individual drugs was detected between clustered and unique isolates. In some cases, members of the same cluster showed different drug susceptibility profiles. For example, the major Beijing cluster, FP25, comprised two fully susceptible, one rifampicin-monoresistant and five MDR strains. Members of the second major Beijing cluster, FP42, shared similar drug susceptibility profiles but had different mutations in the \( rpoB \) gene. These data indicate that this particular strain acquired rifampicin resistance after dissemination. The number of new cases was slightly higher among clustered isolates (29-6 versus 24-3%). Two of eight (25%) unique new cases were MDR.

In some cases, ignoring close similarities between IS6110 patterns may result in underestimation of the epidemiological links between patients (Yeh et al., 1998). It has been shown in a number of studies (Niemann et al., 2000; Yeh et al., 1998; Warren et al., 2002) that up to four IS6110 banding pattern changes (appearance, disappearance or shift of a band) may occur during recent transmission or persistent disease. It has been demonstrated that about 20% of transmission events produced variant strains (van der Spuy et al., 2003). About 31% (27/87) of isolates recovered in the prison hospital shared identical spoligotype ST252, attributed to the LAM09 spoligofamily. According to IS6110 typing, two clusters (FP7 and FP8) and five unique FPs are contained in this group of isolates. The overall similarity in this group was 85%. The genetic distance between the isolates [number of mismatched bands in IS6110 patterns

### Table 3. Mutations in \( rpoB \) of rifampicin-resistant isolates of \( M. \) tuberculosis, according to spoligofamily

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Beijing ( n = 31 )</th>
<th>LAM ( n = 34 )</th>
<th>Other ( n = 1 )</th>
<th>Total ( n = 66 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS11P</td>
<td>1 (3-2%)</td>
<td>0 (0-0%)</td>
<td>0 (0-0%)</td>
<td>1 (1-5%)</td>
</tr>
<tr>
<td>DS16V</td>
<td>0 (0-0%)</td>
<td>27 (79-4%)</td>
<td>0 (0-0%)</td>
<td>27 (40-9%)</td>
</tr>
<tr>
<td>H526Y</td>
<td>1 (3-2%)</td>
<td>0 (0-0%)</td>
<td>0 (0-0%)</td>
<td>1 (1-5%)</td>
</tr>
<tr>
<td>H526D</td>
<td>2 (6-5%)</td>
<td>2 (5-9%)</td>
<td>0 (0-0%)</td>
<td>4 (6-1%)</td>
</tr>
<tr>
<td>SS31L</td>
<td>24 (77-4%)</td>
<td>4 (11-8%)</td>
<td>0 (0-0%)</td>
<td>28 (42-4%)</td>
</tr>
<tr>
<td>Not identified*</td>
<td>3 (9-7%)</td>
<td>1 (2-9%)</td>
<td>1 (100-0%)</td>
<td>5 (7-6%)</td>
</tr>
</tbody>
</table>

*No mutations in the RRDR were detected.
In our study, 44% of them were MDR (Supplementary Table S3 in JMM Online). The Russian Federation has one of the highest incarceration rates in the world. In 2002, there were 640 inmates per 100,000 population (Bobrik et al., 2005). Thus, insufficient TB control measures in prison settings have an substantial impact on communities outside prisons, especially in Russia. Our results showed that, in contrast to other geographical regions of Russia, together with Beijing strains, LAM strains were major contributors to the TB epidemiological picture and accounted for most of the drug-resistant TB in the population studied.

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REFERENCES


