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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The genotypic characteristics and drug susceptibility profiles of clinical isolates of *Mycobacterium tuberculosis* recovered from prison hospital patients in the Tula region (central Russia) during 2001 and 2002 are reported. The emergence of multi-drug-resistant tuberculosis (TB) poses a major health risk to the population, with economic implications for TB control. Prisons serve as a continuous source of TB transmission. The results showed that members of the LAM and Beijing families are major contributors to the epidemiological picture of TB in the population studied. The two families of strains accounted for most of the drug-resistant TB in the population. The genotypic characteristics of the *M. tuberculosis* predominant LAM strain that was responsible for 31 % of TB cases in this setting are presented.

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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. rSfor and rSrev primers were used for sequencing the segment of *rpoB*, and kSfor and kSrev 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 15.1 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 means (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 only 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. Spoligotyping of 87 isolates produced 13 distinct profiles. Spoligopatterns were compared with those published in the International Spoligotyping Database of the Institut Pasteur de Guadeloupe (Brudey *et al.*, 2006). Two isolates (2.3%)

Table 2. Prevalence of drug resistance in new and previously treated cases

INH, Isoniazid; KAN, kanamycin; MDR, multi-drug resistant; RIF, rifampicin; STR, streptomycin.

Drug resistance	New cases (<i>n</i> =24)	Previously treated cases (<i>n</i> =63)	Total (<i>n</i> =87)
Susceptible	6 (25.0 %)	2 (3.2 %)	8 (9.2 %)
STR	17 (70.8 %)	60 (95.2 %)	77 (88.5 %)
INH	12 (50.0 %)	55 (87.3 %)	67 (77.0 %)
KAN	9 (37.5 %)	49 (77.8 %)	58 (66.7 %)
RIF	10 (41.7 %)	56 (88.9 %)	66 (75.9 %)
MDR	9 (37.5 %)	53 (84.1 %)	62 (71.2 %)

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 D516V or S531L 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

Mutation	Beijing (<i>n</i> =31)	LAM (<i>n</i> =34)	Other (<i>n</i> =1)	Total (<i>n</i> =66)
L511P	1 (3.2 %)	0 (0.0 %)	0 (0.0 %)	1 (1.5 %)
D516V	0 (0.0 %)	27 (79.4 %)	0 (0.0 %)	27 (40.9 %)
H526Y	1 (3.2 %)	0 (0.0 %)	0 (0.0 %)	1 (1.5 %)
H526D	2 (6.5 %)	2 (5.9 %)	0 (0.0 %)	4 (6.1 %)
S531L	24 (77.4 %)	4 (11.8 %)	0 (0.0 %)	28 (42.4 %)
Not identified*	3 (9.7 %)	1 (2.9 %)	1 (100.0 %)	5 (7.6 %)

*No mutations in the RRDR were detected.

(van der Spuy *et al.*, 2003)] did not exceed three, thus allowing consideration of this group as a supercluster. These isolates shared drug-resistance patterns. All of them were resistant to streptomycin, rifampicin, isoniazid and kanamycin and all of them carried the mutation D516V in *rpoB* and S315T substitution in *katG*. Retrospective analysis of the isolates recovered in the Serpukhov prison (Shemyakin *et al.*, 2004) showed that 11.5 % (13/113) of them belonged to this supercluster and had identical drug-resistance profiles. The two prisons are 120 km apart. These data support the assumption that a particular parental strain had been circulating in the environment for a prolonged time and had been split into a number of variant strains. The data above suggest that this strain may present a higher risk of transmission with respect to other strains found in the study.

DISCUSSION

Difficulties in establishing transmission links among prisoners are well known (Jones *et al.*, 1999). Only limited clinical information on patients was available in this study and patients' contacts were not identified. However, the high proportion of clustered isolates and the spread of drug resistance in new cases suggest active transmission among the prisoners.

Two families of *M. tuberculosis* strains were predominant among the isolates analysed. The Beijing/W family of strains is known for its frequent association with multi-drug resistance and high emergence worldwide (Glynn *et al.*, 2002). Several outbreaks associated with this family have been reported (Bifani *et al.*, 1996; Milan *et al.*, 2004). The prevalence of Beijing isolates in Russia, both in prisons and in the community, and the high rate of multi-drug resistance typical for this group have been recognized in a number of studies (Toungoussova *et al.*, 2003; Ruddy *et al.*, 2005; Niemann *et al.*, 2000; Shemyakin *et al.*, 2004). Our results were consistent with this observation – 43.7 % (38/87) of isolates were attributed to the Beijing family and 76.3 % (29/38) of them were MDR (Supplementary Table S3 in JMM Online).

Whereas a considerable amount of information is available on the Beijing/W family, the same is not true for other families. Limited data are available on epidemiological and molecular characteristics of the LAM family. Our results together with the data reported previously show that this family is widely spread in central Russia (Shemyakin *et al.*, 2004). In our study, 44.8 % (39/87) of isolates were members of the LAM family. Drug susceptibility testing has revealed a high rate of MDR isolates in this group (33/39, 84.6 %). Fifty seven per cent of the new cases (4/7) and 90.6 % (29/32) of previously treated cases were MDR. Overall, 100 % of MDR isolates among both previously treated and new cases belonged to the Beijing or LAM families (see Supplementary Table S3 in JMM Online).

The high rate of drug-resistant TB in the prison setting is a major concern. Treatment of MDR TB is complex. Patients

with MDR TB remain infectious for a longer time, increasing the probability of spread of infection. Early identification and isolation of MDR TB cases and switching to second-line drug therapy is an important part of a TB control programme. Molecular methods may speed up the diagnosis of drug resistance. The main limitation of any genotypic technique that aims to detect drug resistance is that it can detect only those mutations that are screened for. However, the accurate choice of target mutations enables detection of the majority of resistance. As was shown in a 5 year community-based study of TB in South Africa, more than 90 % of resistance to rifampicin, streptomycin and ethambutol and 75 % of resistance to isoniazid could be detected by assaying six mutations (Van Rie *et al.*, 2001).

An unusually high proportion of the S315T mutation in *katG* is characteristic of isoniazid-resistant strains in Russia (Nikolayevsky *et al.*, 2004). According to previously reported data (Generozov *et al.*, 2000), mutations in codons 516, 526 and 531 of *rpoB* are responsible for 86–100 % of rifampicin-resistant TB cases in Russia and are among the most frequently reported in other regions with high rates of MDR TB (Ramaswamy & Musser, 1998). These mutations were targeted by ASD-PCR. Using this technique, we accurately identified isoniazid-resistant and susceptible isolates (87/87). Rifampicin susceptibility of 81 (93.1 %) and 82 (94.3 %) of 87 isolates was identified correctly by using the ASD-PCR assay and by sequencing, respectively. In comparison with sequencing, only one isolate was not recognized as rifampicin resistant as it contained the substitution in codon 511 of *rpoB* that was not targeted by the ASD-PCR assay. These results demonstrate that the mutations targeted by ASD-PCR were relevant in this setting. More efforts should be made to prevent the spread of MDR TB and early case finding is a crucial part of the prevention strategy. The introduction of molecular methods of MDR TB diagnostics would improve disease management and decrease the transmission rate.

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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