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Molecular epidemiology and prevalence of mutations conferring rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* strains from the southern Ukraine

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ABSTRACT

Understanding the molecular epidemiology of tuberculosis (TB) and mutations in genes associated with drug resistance may contribute to the development of appropriate interventions to improve tuberculosis control. A structured questionnaire was used to collect basic epidemiological data from 589 patients with radiologically confirmed TB in the Odessa and Nikolaev regions of the Ukraine in 2003–2004. A non-commercial reverse hybridisation assay and DNA sequencing were used to detect mutations associated with rifampicin and isoniazid resistance. Genotyping was performed using multilocus variable number tandem repeat (VNTR) typing and spoligotyping. Mutations conferring rifampicin and isoniazid resistance were detected in 32.9% and 44.0%, respectively, of 225 *Mycobacterium tuberculosis* isolates from individual consecutive patients. Mutations in codon 531 and codon 315 of the *rpoB* and *katG* genes, respectively, were predominant among drug-resistant isolates. Multidrug (MDR) resistance rates were significantly higher among former prison inmates compared with non-prisoners (54.8% vs. 27.3%; RR 2.01; 95% CI 1.35–2.97) and the prevalence of mutations was higher in Beijing strains sharing the VNTR signature 223325173533424 than in other Beijing strains (71.4% vs. 45.7%; RR 1.74; 95% CI 1.17–2.57), suggesting that this group may be responsible for rapid transmission of MDR TB in the southern Ukraine.

Keywords Molecular typing, mutations, *Mycobacterium tuberculosis*, resistance, typing, Ukraine

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INTRODUCTION

Tuberculosis (TB) remains one of the major infectious diseases worldwide, causing 2 million deaths annually [1]. In the Ukraine, Russia and the majority of the other states of the former Soviet Union (FSU), the last 15 years have been characterised by a dramatic rise in the incidence of TB and associated mortality in conjunction with a converging human immunodeficiency virus (HIV)/AIDS epidemic [2,3]. In 2003, the mean incidence of TB and mortality rates in the Ukraine had risen to 77.5/100 000 and 21.8/100 000, respectively (Ukrainian TB and Pulmonology Service, 2004 Annual Update; http://www.ifp.kiev.ua). Southern regions, including Odessa and Nikolaev, are hotspots for the TB and HIV epidemics in the Ukraine [3]. Actual rates of both primary and acquired drug resistance are unknown as this information is not officially registered and monitored, but limited retrospective data for selected regions demonstrate a substantial rise in drug resistance rates [4].

Molecular methods of drug resistance analysis, based on the identification of mutations in genes associated with the development of drug resistance, offer an effective tool for drug resistance testing because of their high sensitivity, specificity and speed. The rapid detection of isoniazid,

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rifampicin and multidrug resistance (MDR; resistance to at least isoniazid and rifampicin) is particularly useful for effective treatment and public health control. Several genes, including *katG*, *inhA*, *kasA* and *ahpC*, have been associated with resistance to isoniazid [5–7]. Elsewhere, modifications of the *katG* gene were found in *c*.75–90% of resistant strains, whereas mutations in the *inhA* gene and the *mabA-inhA* regulatory region are less common and are seen in *c*.5–10% of resistant strains [6,7]. In Samara Oblast (central Russia), 90.4% of phenotypically defined isoniazid-resistant strains have been shown to possess mutations in codon 315 of the *katG* gene and the regulatory region of the *inhA* gene [8], demonstrating that other mechanisms (e.g., mutations in *kasA*, *ahpC*, other codons of the *katG* gene, and as yet unknown mechanisms) may be responsible for the development of resistance in <10% of *M. tuberculosis* strains. More than 90% of rifampicin-resistant strains have been shown to possess point mutations in an 81-bp rifampicin resistance-determining region of the *rpoB* gene (RRDR), but the contribution of certain mutations in different codons varies substantially in different countries [5,6,9]. This may affect the performance of molecular assays significantly.

Recently, non-commercial molecular methods for rifampicin and isoniazid susceptibility analysis have been described, based on reverse dot-blot hybridisation. These offer cost-effective and accurate alternatives to commercial kits for research purposes in resource-poor regions with high TB burdens and drug resistance rates [8–11]. However, differing geographical distributions of mutations may demand modification of oligonucleotide probe sets to achieve high sensitivity and specificity of non-commercial assays.

The recent development of PCR-based molecular genotyping methods has led to identification and further differentiation of a number of clinically and epidemiologically important genetic groups of *M. tuberculosis*. Development of high-throughput multilocus techniques, using panels of variable number tandem repeat (VNTR) loci, has offered the possibility of rapid genotyping with a discriminatory power comparable with that of RFLP-IS*6110* typing [12–14]. Spoligotyping, although of limited discriminative capacity compared with RFLP-IS*6110* and VNTR typing, is particularly useful for identification of the Beijing, LAM, Haarlem, CAS and other common families and lineages of *M. tuberculosis* [15]. Beijing family strains have been shown to be responsible for a number of TB outbreaks in the USA [16] and are currently dominant across several Asian countries and in central and north-western Russia [17–20], whereas the LAM and T families are most prevalent in central and south America and Africa [15]. A significant association of Beijing family strains with drug resistance has been reported in studies from Russia, the USA and Europe [16,19,20], but this correlation is weak or absent in several other geographical areas [21]. It is still unclear whether there is a genetic basis for the increased virulence and evolutionary success of the Beijing family. The prevalence of the Beijing and other genotypes of *M. tuberculosis* in the Ukraine is unknown.

The aims of the present study were: (i) to establish the prevalence of mutations associated primarily with isoniazid and rifampicin resistance; (ii) to define the proportion of Beijing and other *M. tuberculosis* strain families circulating in two neighbouring regions in the southern Ukraine using molecular epidemiological tools; and (iii) to determine the association between genotypes and mutations conferring rifampicin and isoniazid resistance in *M. tuberculosis* strains circulating in the southern Ukraine.

MATERIALS AND METHODS

Bacterial isolates

The study was conducted in the Odessa (1 June to 31 July 2003 and 2004) and Nikolaev (in 2003 only, because of organisational changes in patient referral patterns during 2004) regional TB dispensaries that admit all TB cases in the regions. All pulmonary TB patients attending the two dispensaries during the above periods in Odessa and Nikolaev were invited to participate in the study. According to annual regional TB reports, the attendance of patients and the case mix (new and chronic patients) are distributed evenly throughout the year. The study was approved by the Committee for Biomedical Ethics of Odessa State Medical University. Informed consent was obtained from all patients. The overall refusal rate was 11.3%. There were no demographical or clinical differences between recruited patients and those who refused to participate.

Bacteriological methods

Isolation and identification of cultures were performed in the local bacteriological laboratories of the Odessa and Nikolaev TB dispensaries. Sputum specimens were cultured on Lowenstein-Jensen medium. Bacteria belonging to the *M. tuberculosis* complex were identified by their macroscopic and microscopic

appearance, growth characteristics, niacin and nitrate reductase biochemical test results, and by spacer oligonucleotide typing (spoligotyping). A single isolate from each individual patient was analysed.

Molecular drug resistance analysis

Crude DNA extracts were prepared by heating *M. tuberculosis* cell suspensions in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 80°C for 20 min [22]. Molecular drug resistance analysis was performed at the Health Protection Agency (HPA) Mycobacterium Reference Unit (London, UK). Rifampicin and isoniazid resistance analysis was performed using non-commercial macroarrays based on reverse hybridisation of biotin-labelled PCR products to wild-type and mutant oligonucleotide probes immobilised on nylon membranes, as described previously [8,10]. The array included 11 probes for the identification of isolates as members of the *M. tuberculosis* complex and detection of the most frequent mutations in the *rpoB*, *katG* and *inhA* genes. The set of probes for the *rpoB* gene comprised six probes for the detection of wild-type (i.e., no mutation) sequences in appropriate codons, and one probe for the detection of mutations in codon 531. The sets of probes for the *katG* and *inhA* genes both comprised two probes indicating either wild-type sequences or mutations in codons 315 or −15, respectively. Probe sequences used were as described previously [8,10].

Isolates with mutations in the *rpoB* gene were considered to be rifampicin-resistant, and mutations in the *katG* and/or *inhA* genes were considered to be indicative of resistance to isoniazid. Consequently, strains possessing mutations in both *rpoB* and *katG* (or *inhA*) were regarded as MDR. Hybridisation, development and analysis of membranes were performed as described previously [8,10]. The RRDR in the *rpoB* gene was sequenced in isolates designated as rifampicin-resistant in order to identify mutations and to validate the macroarray results. Sequencing was performed as described previously [8].

Genotyping

Spoligotyping was performed as described by Kamerbeek *et al.* [23] using commercially available membranes (Isogen, Maarssen, The Netherlands). Multilocus VNTR analysis was performed using a set of 12 mycobacterial interspersed repetitive units (MIRU) loci and the three exact tandem repeat (ETR) loci (A, B and C) described previously [12,13], with primer sequences as described above [12,24]. Both multiplex and single PCRs were performed, taking into consideration dye-labelling and the expected length of PCR products to allow unambiguous automated calling as described previously [25].

In all cases PCR was performed in 10-μL volumes containing 1 μL 10x PCR buffer with 1.5 mM $MgCl_2$ (Bioline Ltd, London, UK), 0.5 U *Taq* polymerase (Bioline), 0.25 μL 2 mM dNTPs (Bioline), 0.5 μL 20 μM mix of forward and reverse primers, 7.0 μL water and 1 μL DNA extract. Thermal cycling was performed on a Perkin Elmer 9700 Thermocycler (PE Applied Biosystems, Warrington, UK), with 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C and 60 s at 72°C, followed by 5 min at 72°C and a hold at 4°C. Sequencing on a Coulter CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, CA, USA) was performed as described previously [25]. Analysis of PCR fragment length was performed using the CEQ8000 sequencer and proprietary software (Beckman Coulter). Results of VNTR analysis were presented as a 15-digit code. Further analysis of genotyping data was performed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) with categorical (for VNTR-MIRU) or Jaccard (for spoligotyping) variable indices and the UPGMA algorithm. The Hunter-Gaston diversity index (HGDI) was calculated as described previously [26].

RESULTS

In total, 589 new and chronic patients with radiologically and clinically confirmed TB were recruited (292 in 2003, with 147 in Odessa and 145 in Nikolaev, and 297 in 2004). Key baseline epidemiological data are summarised in Table 1.

Viable *M. tuberculosis* cultures were isolated from 231 patients, giving an overall isolation rate of 39.2% (Table 1). Two-thirds (65.6%) of isolates were cultured from patients who had never been treated for TB. Overall, 16.0% (n = 37) of cultures were isolated from former prison inmates, and 3.9% (n = 9) from homeless patients. Nearly all patients were ethnic Russians or Ukrainians. All cultures were identified as *M. tuberculosis* by means of standard bacteriological and biochemical methods. Six (2.6%) isolates displayed double-figure values of tandem repeats in several loci following VNTR-MIRU analysis, and were therefore considered to be mixed cultures. These were excluded from further analysis (leaving 225 patient isolates).

Prevalence of mutations associated with drug resistance

The results of molecular analysis of *M. tuberculosis* isolates from patients with newly diagnosed TB

Table 1. Baseline demographical and clinical parameters of patients

	All cases		New cases		Previously treated cases	
Parameters	**n = 589**	**%**	**n = 392**	**%**	**n = 197**	**%**
Gender						
Male	465	78.9	294	75.0	171	86.8
Female	124	21.1	98	25.0	26	13.2
Age/years (median)	38	–	33	–	43	–
Residence						
Homeless	13	2.2	5	1.3	8	4.1
Urban-dwellers	156	26.5	108	27.6	48	24.5
Rural	419	71.3	279	71.1	140	71.4
Previously in prison	82	13.9	33	8.4	49	25.0
Smear-positive	219	37.2	139	35	80	40.6
Culture-positive[a]	231	39.2	144	36.7	87	44.2

[a]Six mixed cultures were excluded from further molecular analysis, i.e., analysis was performed with 225 patients.

and those treated previously are shown in Tables 2 and 3. The prevalence of mutations associated with rifampicin and isoniazid resistance was significantly higher among isolates from previously treated patients. Fifty-nine (70.2%) isolates from patients with a history of anti-TB treatment harboured mutations in the *katG* and/or *inhA* genes, indicating resistance to isoniazid. The proportions of isolates with mutations associated with rifampicin resistance were slightly lower, comprising 16.3% and 60.7% of new and chronic cases, respectively. Twenty (14.2%) isolates from patients with newly diagnosed TB had mutations in both *rpoB* and *katG* (or *inhA*), and were therefore considered to be MDR. No statistically significant difference was observed between the rates of mutations in strains circulating in the Odessa and Nikolaev regions, or between 2003 and 2004 (data not shown). In contrast, the proportion of isolates from former

Table 2. Prevalence of Beijing strains and mutations associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* isolates from patients in Nikolaev (2003) and Odessa (2003–2004) regions in the southern Ukraine

		Nikolaev (n = 42)			Odessa (n = 183)			All (n = 225)		
Genes affected	**Codons evaluated**	**New cases**	**Previously treated cases**	**Total**	**New cases**	**Previously treated cases**	**Total**	**New cases**	**Previously treated cases**	**Total**
Rifampicin resistance[a]		5/23 21.7%	12/19 63.2%	17/42 40.5%	18/118 15.3%	39/65 60.0%	57/183 31.1%	23/141 16.3%	51/84 60.7%	74/225 32.9%
RpoB probe 3	509–516	0	0	0	0	1/39 2.6%	1/57 1.8%	0	1/51 2.0%	1/74 1.4%
RpoB probe 6	512–519	1/5 20.0%	1/12 8.3%	2/17 11.8%	3/18 16.7%	3/39 7.7%	6/57 10.5%	4/23 17.4%	4/51 7.8%	8/74 10.8%
RpoB probe 9	515–522	0	0	0	0	0	0	0	0	0
RpoB probe 12	518–525	0	0	0	0	0	0	0	0	0
RpoB probe 17	523–529	3/5 60.0%	7/12 58.3%	10/17 58.8%	2/18 11.1%	3/39 7.7%	5/57 8.8%	5/23 21.7%	10/51 19.6%	15/74 20.3%
RpoB probe 22	528–534	1/5 20.0%	4/12 33.3%	5/17 29.4%	14/18 77.8%	33/39 84.6%	47/57 82.5%	15/23 65.2%	37/51 72.5%	52/74 70.3%
Isoniazid resistance		9/23 39.1%	12/19 63.2%	21/42 50.0%	31/118 26.3%	47/65 72.3%	78/183 42.6%	40/141 28.4%	59/84 70.2%	99/225 44.0%
katG only	315	6/9 66.7%	9/12 75.0%	15/21 71.4%	15/31 48.4%	38/47 80.9%	53/78 67.9%	21/40 52.5%	47/59 79.7%	68/99 68.7%
inhA only	− 15 (regulatory region)	1/9 11.1%	0	1/21 4.8%	5/31 16.1%	0	5/78 6.4%	6/40 15.0%	0	6/99 6.1%
Both *katG* and *inhA*	As above	2/9 22.2%	3/12 25.0%	5/21 23.8%	11/31 35.5%	9/47 19.1%	20/78 25.6%	13/40 32.5%	12/59 20.3%	25/99 25.3%
MDR	As above	4/23 17.4%	11/19 57.9%	15/42 35.7%	16/118 13.6%	39/65 60.0%	55/183 30.1%	20/141 14.2%	50/84 59.5%	70/225 31.1%
Beijing strain prevalence		6/23 26.1%	6/19 31.6%	12/42 28.6%	37/118 31.4%	40/65 61.5%	77/183 42.1%	43/141 30.5%	46/84 54.8%	89/225 39.6%

[a]Some isolates had two codons affected in the *rpoB* gene.

Table 3. Association of genotypes with mutations conferring drug resistance in *Mycobacterium tuberculosis* isolates from the southern Ukraine

	Genotype					
Drug resistance pattern and mutations	**Beijing 1 223325153533424**	**2 223325173533424**	**3 Other patterns**	**Non-Beijing**	**Beijing to non-Beijing RR (95% CI)**	**Subgroup 2 to all other Beijing strains RR (95% CI)**
Mutations conferring INH resistance	18/35 51.4%	19/21 90.5%	15/33 45.5%	47/136 34.6%	1.69[b] (1.26–2.28)	1.86[b] (1.41–2.47)
Mutations conferring RIF resistance	16/35 45.7%	15/21 71.4%	12/33 36.6%	31/136 22.8%	2.12 [b] (1.45–3.09)	1.73[b] (1.17–2.57)
Mutations conferring multidrug resistance	16/35 45.7%	15/21 71.4%	12/33 36.6%	27/136 19.9%	2.43 [b] (1.63–3.63)	1.74[b] (1.17–2.57)
No mutations	17/35 48.6%	2/21 9.5%	18/33 54.5%	81/136 59.6%	0.70 [b] (0.53–0.93)	0.19 [b] (0.05–0.71)
Mutations in *rpoB* gene codon 531	13/16[a] 81.3%	13/15 [a] 86.7%	9/12 [a] 75.0%	16/31 [a] 51.6%	1.58 [b] (1.09–2.28)	1.10 (0.84–1.46)
Mutations in *katG* gene codon 315	18/18 [a] 100%	19/19 [a] 100%	15/15 [a] 100%	41/47 [a] 87.2%	1.15 [b] (1.03–1.28)	– –
Mutations in *inhA* gene regulatory region	9/18 [a] 50.0%	0/19 [a] –	0/15 [a] –	22/47 [a] 46.8%	0.37 [b] (0.19–0.72)	– –

[a]Proportion of strains resistant to corresponding drug.
[b]Statistically significant difference (95% CI).
INH, isonazid; RIF, rifampicin.

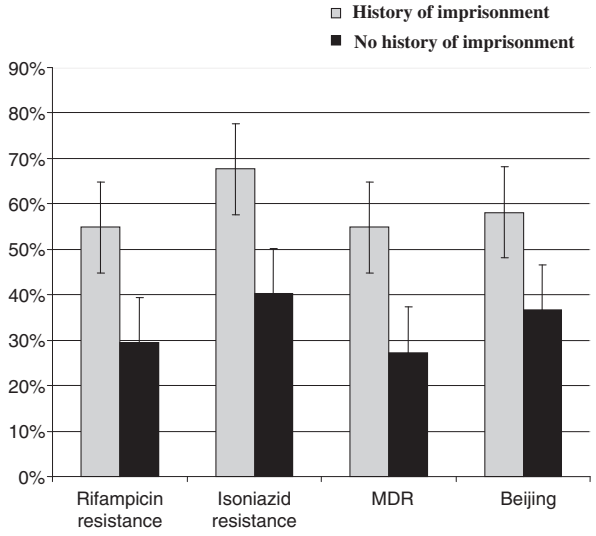

Fig. 1. Prevalence of mutations associated with drug resistance and Beijing family strains among former prison inmates. MDR, multiple drug resistance.

prison inmates that harboured mutations was significantly higher compared with that in individuals who had never been in prison (RR 1.67, 95% CI 1.15–2.41; RR 1.54, 95% CI 1.16–2.05; and RR 1.67, 95% CI 1.15–2.41, respectively) (Fig. 1).

Sequencing of the *rpoB* gene RRDR in 74 isolates that displayed negative hybridisation with one or more wild-type macroarray probes, and were therefore considered to be rifampicin-resistant, allowed precise identification of codons affected by mutations. The results of sequencing and macroarray analysis were concordant for all isolates. Seventy-one (95.9%) isolates possessed mutations that have been reported previously to be associated with high levels of rifampicin resistance (i.e., S531L, S531W, H526Y, H526N, H526D and D516V) [27]. Two isolates had mutations associated with a low level of rifampicin resistance (codon 514 duplication and L533P). The mutation G523W, which has been reported to be associated with rifampicin resistance [27], was found in one isolate.

The diversity of genes and codons affected by mutations associated with rifampicin and isoniazid resistance in *M. tuberculosis* strains from Odessa and Nikolaev is summarised in Table 2. Mutations in codon 315 of the *katG* gene were found in 93 (93.9%) of 99 isolates with mutations associated with resistance to isoniazid, and in 26.9% of these isolates, mutations in the *katG* gene were combined with mutations in the regulatory region of the *inhA* gene. Mutations only in the *inhA* gene were seen in six isolates (6.1% of the isolates with mutations associated with resistance to isoniazid).

Single mutations were observed in the vast majority (97.3%) of isolates with mutations in the RRDR, with mutations in codon 531 dominating. In Odessa, the proportion of mutations in codon 531 was significantly higher than in Nikolaev (80.7% vs. 29.4%; RR 2.7; 95% CI 1.3–5.8), while mutations in codon 526 were more frequent in Nikolaev (RR 11.1; 95% CI 4.8–25.8). Double mutations in the *rpoB* gene (in codons 516 and 531, and in codons 526 and 531, respectively) were detected in two isolates.

Spoligotyping

Spoligotyping of 225 *M. tuberculosis* isolates yielded 21 clustered isolates (containing between two and 75 isolates with identical profiles) and 52 individual patterns. The number of clustered isolates in this panel was 173 (76.9% of all isolates). The diversity of the spoligotyping profiles is summarised in Fig. 2. Eighty-nine (39.6%) isolates with missing spacers 1–34 in the spoligotyping pattern were considered to belong to the Beijing family. However, the most common Beijing profile (absence of spacers 1–34 and presence of spacers 35–43) was seen in only 75 isolates (comprising the largest cluster of spoligotyping patterns), with incomplete profiles observed for 14 other isolates. In these latter isolates, spacers 37 and 35 were missing in eight isolates and one isolate, respectively. In the remaining five isolates, four lacked spacers 37 and 38, and one lacked spacers 38, 39 and 40. Spoligotyping also allowed the identification in the Odessa and Nikolaev regions of other known *M. tuberculosis* genotypes. These included the LAM family (with the largest cluster containing seven LAM-9 isolates), the T1 group (17 isolates) and the Haarlem family (with the largest cluster containing 13 isolates).

Statistical analysis demonstrated that Beijing strains dominated in patients with a known history of anti-TB treatment (54.8% vs. 30.5% in new cases; RR 1.79, 95% CI 1.31–2.46), and were also more prevalent among former prison inmates compared with TB patients with no known history of imprisonment (58.1% vs. 39.6%; RR 1.47, 95% CI 1.04–2.06) (Table 2 and Fig. 1).

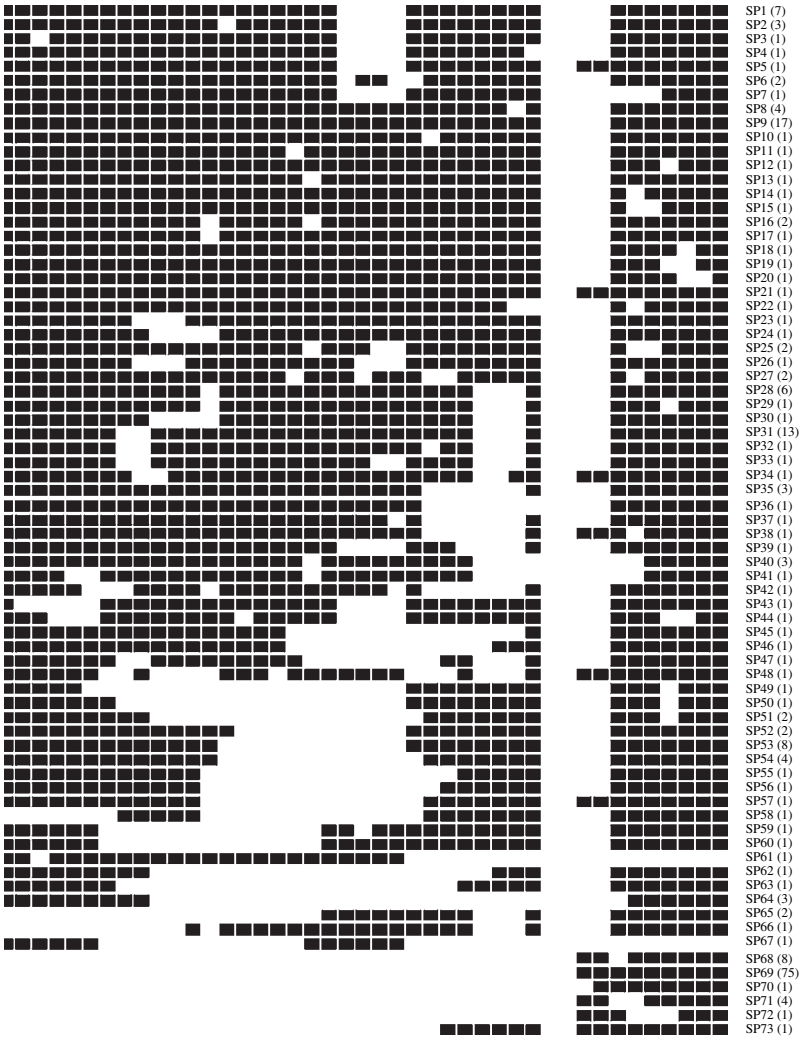

Fig. 2. Computer-generated image showing 73 variants of spoligopatterns of *Mycobacterium tuberculosis* isolates from Odessa and Nikolaev. SP1 to SP73 are variants of spoligopatterns; numbers in parentheses indicate the number of isolates sharing each spoligotype.

Analysis of prevalence of mutations in Beijing and non-Beijing strains

The prevalence of mutations associated with all types of resistance was significantly higher among Beijing strains, with the strongest association being with MDR (48.3% vs. 19.8%; RR 2.43, 95% CI 1.63–3.63) (Table 3). Drug susceptibility was more common among non-Beijing strains (RR 1.43, 95% CI 1.08–1.90). Substantial differences were also observed in the distribution of mutations among Beijing and non-Beijing strains; 46.8% of non-Beijing strains harboured mutations in the regulatory region of *inhA*, whereas the prevalence of such mutations in Beijing isolates was much lower (RR 2.70, 95% CI 1.39–5.27). Mutations in codon 531 of *rpoB* were more common among Beijing strains (RR 1.58, 95% CI 1.09–2.28) and less frequent among other groups of isolates. No significant differences in the distribution of mutations in other regions of *rpoB* was seen.

Multilocus VNTR typing

Multilocus VNTR genotyping of 225 *M. tuberculosis* isolates using 12 MIRU and three ETR loci yielded 121 distinct genotypes. Overall, 136 (60.4%) isolates formed 31 clusters with 90 individual patterns (compared with 21 clusters and 52 individual isolates using spoligotyping). As expected, the overall discriminatory ability of VNTR genotyping for the Ukrainian isolates was higher than that provided by spoligotyping (HGDI 0.968 vs. 0.878). However, VNTR genotyping of 15 loci was unable to discriminate within the Beijing family isolates; 68.6% of Beijing family isolates comprised two large clusters formed of 35 and 21 isolates sharing the MIRU-ETR signatures

223325153533424 and 223325173533424, respectively, supporting previous reports on the predominance of these genotypes among Beijing isolates in Russia [20,28]. Isolates with the MIRU-ETR signature 223325173533424 were more common among previously imprisoned patients.

Analysis of the prevalence of mutations conferring drug resistance within the Beijing family has demonstrated an uneven distribution of mutations among different genetic subgroups. The prevalence of mutations associated with rifampicin and isoniazid resistance was much higher among isolates with the VNTR signature 223325173533424 than among isolates from other subgroups (RR 1.86, 95% CI 1.41–2.47, and RR 1.74, 95% CI 1.17–2.57, for isoniazid and rifampicin resistance, respectively) (Table 3). In contrast, the prevalence of mutations in isolates sharing genotype 223325153533424 and other profiles in Beijing family isolates was closer to that for non-Beijing isolates, with no significant difference between non-Beijing isolates and Beijing isolates not sharing the predominant signatures 223325153533424 and 223325173533424. Another interesting phenomenon was that mutations in the regulatory region of *inhA* were detected only in eight *M. tuberculosis* isolates sharing profile 223325153533424 (i.e., with five repeats in the locus MIRU26) and an 'incomplete' spoligotyping profile with spacer 37 missing. None of the other 34 isoniazid-resistant Beijing isolates possessed this mutation.

Results of allelic diversity analysis for 15 MIRU and ETR loci demonstrated that the number of allelic variants at MIRU loci varied substantially, with the minimum variability at locus MIRU24 (two variants) and the maximum variability at locus 10 (ten variants). The highest discriminatory power (based on calculation of the HGDI) was observed for locus MIRU31 (HGDI 0.695). This locus, together with other loci displaying HGDIs exceeding 0.6, was regarded as highly polymorphic on the basis of recently proposed definitions [29]. In the panel of ETR loci, the highest discriminatory power was observed for locus ETR-A (HGDI 0.652).

DISCUSSION

This cross-sectional study is the first survey to address the molecular epidemiology of *M. tuberculosis* and drug-resistant *M. tuberculosis* strains circulating in the Ukraine. Drug resistance was determined by detecting mutations associated with resistance to rifampicin and isoniazid. This concept has proven effective for the evaluation of drug resistance, with >90% sensitivity for isoniazid and rifampicin resistance [8,10,30,31]. In Russia, a country with the same approach to TB treatment and the same organisational structure, a good correlation between phenotype and the same molecular analytical assay system has been demonstrated, in which the system was able to detect 95.4% of rifampicin resistance and 90.4% of isoniazid resistance when compared with phenotypic analysis (assuming that culture-based drug resistance detection was definitive) [8]. As *M. tuberculosis* isolates were obtained from consecutive patients visiting two central regional TB dispensaries (referral sites for corresponding regions) during defined periods, it was possible to estimate the extent of drug resistance, with certain limitations, in the southern Ukraine. The culture positivity rates (39.2%) in the current study were similar to those reported from other FSU states [32] and reflect the national guidelines, i.e., diagnosis of TB is often made on the basis solely of radiological abnormalities.

In the present study, 14.2% of isolates from new TB patients possessed mutations associated with MDR TB (there may be higher rates of phenotypically defined drug resistance). This is greater than the primary MDR rates seen in other eastern European countries (i.e., Poland, Hungary, Moldova and the Baltic states) [33,34], and is close to the rates reported for Russia and middle Asia [19,20,35]. Estimation of current drug resistance rates on the basis of molecular analysis, as in the present study, although not validated locally against bacteriological analysis, demonstrated a high prevalence of rifampicin, isoniazid and MDR strains in the southern Ukraine. The proportion of isolates possessing mutations associated with drug resistance was significantly higher among isolates from former prisoners, suggesting that prisons play a significant role in sustaining the TB epidemic. The sensitivity and specificity of the non-commercial macroarrray technique used in the present study was confirmed by sequencing the relevant *rpoB* gene fragment, with complete concordance of results obtained by two methods.

In countries with a high incidence of TB, the spectrum of mutations associated with drug

resistance has been reported to be less diverse than in low-incidence areas, probably because of pronounced genetic homogeneity of strains, dominance of certain genotypes, and high rates of recent transmission [36]. In the present study, the proportion of isolates with mutations in *rpoB* gene codons (67.6%, 18.9% and 8.1% in codons 531, 526 and 516, respectively, with 4.1% of isolates affected by mutations in other codons) was similar to that reported for countries with medium-to-high rifampicin resistance rates and a substantial prevalence of Beijing family strains [37]. Interestingly, the distribution of mutations in *rpoB* gene codons was much more heterogenous than in central Russia, where mutations in codon 531 comprised 90.1% of rifampicin-resistant isolates [10], which may be explained by the preponderance of TB cases among prison inmates, with Beijing strains being predominant. The results of the present study provide further evidence of a strong association between Beijing strains with MDR and mutations in codon 531 of the *rpoB* gene [38] and codon 315 of the *katG* gene [39], as their prevalence was significantly higher among Beijing strains than among other isolates (RR 1.88, 95% CI 1.09–2.28, for mutations in *rpoB*).

In the present study, 31 (31.3%) of 99 isolates with mutations associated with isoniazid resistance possessed transitions at the −15 position in the regulatory region of the *mabA-inhA* locus in *inhA*, which is higher than reported previously [40]. Mutations in *inhA* were much more common among non-Beijing isolates (46.8% vs. 17.3%; RR 2.7; 95% CI 1.39–5.27), but the reason for this is unclear. In Beijing family isolates, mutations in the *inhA* gene were limited to the separated subgroup of nine isolates that shared a specific spoligotype (missing spacer 37) and MIRU-ETR signature 223325153533424. As has been argued by Mokrousov *et al*. [41], spacers 37–38 may be concealed by the presence of IS*6110* insertions in the drug resistance region, preventing primers from binding to the corresponding targets, but an association between these specific spoligotypes and mutations in *inhA* has not been reported previously. Spoligotyping results demonstrated a relative diversity in the genetic structure of *M. tuberculosis* strains circulating in the southern Ukraine, with a significant prevalence of known *M. tuberculosis* families, including the LAM, T and Haarlem families. Beijing family strains accounted for 39.6% of the total, which is lower than reported previously for selected regions in Russia [19,20]. Therefore, high drug-resistance rates in Odessa, which is a major sea-port, resort and university centre in the Ukraine, may be explained not only by the high prevalence of Beijing strains, but also by the exogenous introduction of drug-resistant strains from high-incidence areas because of higher migration rates, which is in contrast to more isolated areas such as Samara and Archangelsk in Russia.

The evaluation of the discriminatory power of two PCR-based genotyping methods for the differentiation of *M. tuberculosis* strains circulating in the southern Ukraine demonstrated a high discriminative ability for multilocus VNTR typing, using the panel of 12 MIRU and three ETR loci, with an overall HGDI of 0.968. Five loci (MIRU10, MIRU26, MIRU31, MIRU40 and ETR-A), with a HGDI of > 0.6 and profound allelic diversity (5–10 allelic variants), were considered to be highly discriminatory, based on the definition of Sola *et al*. [29]. In comparison with the results of a recent molecular epidemiological study in the Samara region (personal unpublished data), in which none of the 15 MIRU-ETR loci was identified as being highly polymorphic, it seems that the population of *M. tuberculosis* strains in the southern Ukraine is more heterogenous and less clonal. However, the present results also support recent evidence that the discriminatory power of multilocus VNTR typing, using the panel of 15 loci for *M. tuberculosis* isolates, is compromised in populations with a high prevalence of Beijing family strains [28]; therefore, introduction of an enlarged panel of highly polymorphic VNTR loci could improve discrimination significantly.

Further differentiation of Beijing strains using multilocus VNTR typing allowed the identification of a number of subtypes within the Beijing family that showed significant disequilibrium in distribution of mutations conferring drug resistance. The prevalence of mutations in isolates sharing genotype 223325173533424 was significantly higher than in other genotypes, suggesting unequal association of different clones with drug resistance within the Beijing family. This finding may explain disagreements in published reports with respect to the association between Beijing strains and drug resistance in different geographical settings [19–21,42]. On the basis of a recently reported association of strains sharing genotype 223325173533424 with imprisonment [20], sup-

ported by the relatively higher proportion of these strains among isolates from previously imprisoned TB patients that was revealed by the present study, it can be speculated that this particular genotype may have evolutionary advantages that may be responsible for rapid transmission of MDR *M. tuberculosis* isolates in Russia and the Ukraine. The genetic basis for this association, as well as the factors primarily responsible for the transmission of drug-resistant Beijing strains among civilians and former prisoners, has yet to be identified.

In conclusion, this first snapshot study in the southern Ukraine, although mainly laboratory-focused, demonstrated a high prevalence of mutations conferring isoniazid and rifampicin resistance, and established relationships among drug resistance patterns, genotypes and selected epidemiological parameters. Clearly, there is a need for larger prevalence studies aimed at detecting overall drug resistance rates and major risk-factors for TB, and at mapping drug-resistant TB transmission. This information is crucial in planning treatment and prophylaxis for both immunocompetent TB patients and TB patients with HIV co-infection, who are becoming increasingly prevalent in the southern Ukraine.

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