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Risk factors for drug-resistant tuberculosis patients in Lithuania, 2002–2008

To the Editors:

Lithuania, a high-priority country for tuberculosis (TB) control in the World Health Organization European Region, has one of the world's highest rates of multidrug-resistant (MDR)-TB. It has recently seen an increase in the rates of both primary and acquired MDR-TB (9% of new and 50% of re-treatment cases were MDR in 2010), and the appearance of extensively drug-resistant (XDR)-TB cases constituting 4.3% of all MDR-TB cases [1, 2]. Drug resistance is accompanied by low treatment success rates (40% in newly diagnosed and 19% in re-treatment cases in 2009) among MDR-TB patients despite a well-established TB control programme with relatively good indicators of treatment success and low default rates (7%) among patients with sensitive TB [2].

Although there are data describing the molecular epidemiology of drug resistance in Lithuania [3], relatively little is known about risk factors for drug resistance. We analysed 7 yrs of Lithuanian national surveillance data: all treated culture-confirmed TB cases, including new and re-treatment cases, registered from 2002 to 2008 in the national TB register (established in 2002). Our aim was to describe the epidemiological, clinical and socioeconomic features of MDR-/XDR-TB cases, and to establish risk factors for drug resistance acquisition and development during re-treatment.

Standard case reporting included demographic and clinical information with initial and follow-up drug susceptibility testing (DST) results. Individual patients suspected of having a high risk of HIV/AIDS were offered testing for HIV according to the national policy. A randomly selected proportion of strains (~18%) was genotyped (by IS6110 restriction fragment length polymorphism typing and spoligotyping) within the routine service by the Lithuanian Institute of Biotechnology (Vilnius, Lithuania).

Non-MDR-TB patients who received a second treatment cycle, and were reported as susceptible to isoniazid and rifampicin in

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this treatment cycle, were defined as ''non-MDR-TB patients that remained non-MDR-TB''. Similarly, non-MDR-TB patients receiving a second treatment cycle and who were reported as MDR by subsequent DST were defined as ''non-MDR-TB patients with acquired MDR-TB''. Non-MDR-TB patients who subsequently developed XDR-TB determined by follow-up DST were defined as ''non-MDR-TB patients who acquired XDR-TB''.

All analyses were performed using STATA version 11 (StataCorp, College Station, TX, USA).

The project was reviewed by the Vilnius Regional Committee for Biomedical Research Ethics (Vilnius University, Vilnius, Lithuania) and Queen Mary College Research Ethics Committee (University of London, London, UK). Patient consent was not required.

From 2002 to 2008, a total of 10,664 culture-confirmed TB cases with available DST results were reported in Lithuania; 2,074 had been treated before 2002 with no data on their first treatment cycle and so were excluded from the analysis. Of 8,590 included patients, 7,833 (91.2%) cases were defined as non-MDR-TB, 729 (8.5%) cases were MDR-TB and 28 (0.3%) were XDR-TB. Of the 7,833 patients who were non-MDR at baseline, 752 had at least two completed treatment cycles and full data on all variables of interest at the first and second treatment cycles; 164 (21.8%) had

TABLE 1 Predictors of primary multidrug and extensive drug resistance in tuberculosis (TB) patients registered for treatment in Lithuania between 2002 and 2008: results of multivariable logistic regression

Characteristics	XDR-TB	MDR-TB	Non-MDR-TB	MDR-/XDR-TB *versus* non-MDR-TB		XDR-TB *versus* MDR-TB	
				OR[#] (95% CI)	p-value	OR[#] (95% CI)	p-value
Patients n	28	729	7833				
Males	20 (71.4)	540 (74.1)	5596 (71.4)	1.01 (0.83–1.24)	0.89	1.24 (0.44–3.66)	0.69
Age yrs							
<30	4 (14.3)	140 (19.2)	1178 (15.0)	1.00	<0.001	1.00	0.71
30–39	9 (32.1)	175 (24.0)	1584 (20.2)	0.76 (0.59–0.98)		2.22 (0.62–7.94)	
40–49	7 (25.0)	192 (26.3)	2084 (26.6)	0.62 (0.48–0.81)		1.59 (0.40–6.29)	
50–59	4 (14.3)	144 (19.8)	1434 (18.3)	0.67 (0.51–0.88)		1.13 (0.25–5.07)	
⩾60	4 (14.3)	78 (10.7)	1553 (19.8)	0.44 (0.32–0.61)		1.59 (0.31–8.30)	
Urban living	19 (67.9)	478 (65.6)	4473 (57.1)	1.46 (1.23–1.73)	<0.001	1.24 (0.53–2.91)	0.62
Contact with TB	5 (17.9)	67 (9.2)	405 (5.2)	1.73 (1.30–2.29)	<0.001	2.32 (0.80–6.73)	0.12
Smoking	18 (64.3)	536 (73.5)	5085 (64.9)	0.94 (0.75–1.17)	0.56	0.61 (0.22–1.70)	0.34
Alcohol use							
None	4 (14.3)	91 (12.5)	1572 (20.1)	1.00	<0.001	1.00	0.66
Sometimes	10 (35.7)	257 (35.3)	3274 (41.8)	1.27 (0.96–1.68)		1.07 (0.28–4.10)	
Often	11 (39.3)	333 (45.7)	2775 (35.4)	1.96 (1.44–2.66)		1.18 (0.26–5.35)	
Alcoholic	3 (10.7)	48 (6.6)	212 (2.7)	3.34 (2.15–5.20)		2.65 (0.39–18.0)	
Drug abuse	0 (0.0)	18 (2.5)	39 (0.5)	1.77 (0.89–3.54)	0.11		
Homelessness	1 (3.6)	44 (6.0)	190 (2.4)	1.31 (0.89–1.92)	0.25	0.48 (0.06–4.06)	0.50
Unemployment[¶]	21 (75)	567 (77.8)	5889 (75.2)	1.21 (0.99–1.49)	0.07	0.95 (0.34–2.65)	0.92
Education							
Less than primary[+]	3 (10.7)	48 (6.6)	961 (12.3)	1.00	0.07	1.00	0.65
Primary[+]/secondary[§]	17 (60.7)	519 (71.2)	5263 (67.2)	1.10 (0.77–1.55)		0.53 (0.11–2.58)	
Tertiary[ƒ]	8 (28.6)	162 (22.2)	1609 (20.5)	1.37 (0.94–1.99)		0.72 (0.13–3.91)	
Comorbidity	0 (0.0)	12 (1.6)	136 (1.7)	0.91 (0.48–1.72)	0.77		
TB type							
Pulmonary	27 (96.4)	682 (93.6)	7116 (90.8)	1.00	0.011		
Extrapulmonary	0 (0.0)	12 (1.6)	333 (4.3)	0.40 (0.22–0.74)			
Both	1 (3.6)	35 (4.8)	384 (4.9)	0.84 (0.57–1.22)			
Smear positivity	18 (64.3)	540 (74.1)	5732 (73.2)	0.82 (0.68–1.00)	0.046	0.59 (0.25–1.41)	0.24
Cavity	19 (67.9)	494 (67.8)	4857 (62.0)	1.06 (0.92–1.32)	0.31	1.24 (0.52–2.98)	0.62
Strain family							
Non-Beijing	3 (10.7)	76 (10.4)	269 (3.4)				
Beijing	6 (21.4)	49 (6.7)	47 (0.6)			1.24 (0.44–3.66)	0.69
Unknown	19 (67.9)	604 (82.9)	7517 (96.0)				

Data are presented as n (%), unless otherwise stated. XDR: extensively drug-resistant; MDR: multidrug-resistant. Non-MDR-TB includes drug-sensitive, and mono- and polyresistant TB. [#]: results of multivariate analysis; [¶]: cases with any known employment are the reference group; [+]: primary school education; [§]: high school education; [ƒ]: college or university education.

acquired MDR, 20 (2.7%) of these had acquired XDR and 568 (75.5%) remained non-MDR.

The typical MDR-/XDR-TB case was a young Lithuanian-born urban-dwelling male, who was unemployed and abused alcohol, had primary or secondary education, reported a TB contact and was infected with a Beijing family TB strain. Drug abuse was relatively uncommon. Most patients had pulmonary disease and were smear-positive at diagnosis. Over 60% of patients had extensive lung damage with cavities identified on radiographs (table 1). Information on time to culture conversion at the second treatment cycle was available for 286 out of 758 patients who were non-MDR at baseline. Culture conversion time varied between 1 and 9 months (median 2 months) in patients who remained non-MDR, 1 and 12 months (median 4 months) in patients who acquired MDR, and 2 and 7 months (median 3 months) in patients who acquired XDR-TB. In total, 328 (3.8%) patients were tested for HIV and 42 (12.8%) of those were positive. HIV status was not included in the multivariable analysis due to small frequencies.

Younger age, urban living, known TB contact and alcohol abuse were all independently associated with increased risk of primary infection with an MDR-/XDR-TB strain. Smear positivity at diagnosis and extrapulmonary TB were associated with infection with non-MDR-TB strains (table 1). In the subsample of patients with genotyped TB strains, in a univariate analysis, there was evidence that a Beijing strain was associated with increased risk of infection with MDR-/XDR-TB (OR 4.00, 95% CI 2.30–6.97; $p<0.001$).

For the comparison of XDR-TB and MDR-TB, some factors (drug abuse, comorbidity and TB type) were excluded from the multivariable analysis because of small numbers in some categories, which predicted outcome perfectly. None of the other examined factors was predictive of increased risk of primary infection with XDR- *versus* MDR-TB strains (table 1).

Unemployment (OR 2.28, 95% CI 1.21–4.32; $p=0.011$), smear positivity at the second treatment cycle (OR 3.18, 95% CI 1.99–5.10; $p<0.001$) and primary mono/polyresistance (OR 1.60, 95% CI 1.02–2.52; $p=0.041$) were all independently associated with increased risk of acquiring MDR-/XDR-TB during treatment in the multivariate analysis. There was weak evidence that a Beijing strain was associated with increased risk of acquiring MDR-/XDR-TB (OR 3.64, 95% CI 0.83–15.9; $p=0.086$) although it just failed to reach statistical significance. While urban residents were at higher risk of acquiring resistant TB, perhaps due to overcrowding, the risk of drug resistance development during treatment did not differ between rural and urban citizens, reflecting common national treatment strategies.

In the comparison of acquired XDR-TB with acquired MDR-TB, known TB contact (OR 11.2, 95% CI 1.10–14.6; $p=0.041$) and absence of cavities when first diagnosed (OR 0.10, 95% CI 0.02–0.49; $p=0.005$) were both independently associated with increased risk of acquiring XDR-TB. Age had a U-shaped relationship with risk of acquiring XDR, the lowest risk being in the 40–49-yr age group. The age and sex distribution might represent a major social problem of alcohol abuse in the male population with long-term chronic alcoholism contributing to a chaotic lifestyle limiting successful therapy and, subsequently, to an increased risk of acquiring XDR-TB in the older group. Problems in the past that have now been solved, such as lack of accurate second-line DST, limited availability of second-line drugs and variable treatment strategies, might be another explanation.

According to the univariate analysis, a longer time to culture conversion was associated with a greater risk of acquiring MDR- or XDR-TB (OR per month 1.75, 95% CI 1.40–2.18; $p<0.001$). It was not associated with risk of acquiring XDR-TB compared with the risk of acquiring MDR (OR per month 0.88, 95% CI 0.60–1.29; $p=0.52$). Key risk factors for MDR-/XDR-TB primary infection and development can be found in the online supplementary material. This is the first national study in Lithuania to examine risk factors for drug resistance in a large cohort of TB patients, complementing smaller studies in other Baltic states [4–6] with similar findings. It showed that a large proportion of all newly diagnosed TB patients develop MDR-TB, contributing to the creation of a pool of infectious patients facilitating further transmission. As initial acquisition and development of drug resistance is strongly associated with social factors, such as alcohol use and unemployment, addressing issues of social support and alcohol dependency is crucial to achieve higher adherence and cure rates. Expansion of HIV testing is necessary for identification of co-infected cases and timely administration of antiretroviral treatment.

The strong association of MDR-/XDR-TB disease and infection with the Beijing strain family supports findings from other studies in Eastern Europe, including Russia [7, 8], Estonia [9] and Latvia [10]. Due to the relatively few genotyping results, we were unable to analyse the relationship between infection with Beijing family TB and XDR-TB. Detailed studies are needed to address this question.

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Early tuberculosis treatment monitoring by Xpert® MTB/RIF

To the Editors:

Progress made to improve laboratory capacity for tuberculosis (TB) diagnosis led to the development of molecular assays that are now replacing conventional microscopy and culture-based methods on a large scale [1, 2]. Unfortunately, current molecular techniques detect both live and dead bacteria, and a positive result does not imply the viability of the pathogen. Indeed, DNA can persist for a long period after bacterial death and nucleic acid from dead bacteria is equally amplifiable. Therefore, molecular assays are unsuitable for treatment monitoring and/or for infection control purposes.

We report an innovative approach to selectively amplify DNA derived from viable *Mycobacterium tuberculosis* in clinical specimens, which is useful for monitoring mycobacterial load in pulmonary TB patients during anti-TB treatment.

The protocol is based on pre-treatment of samples with propidium monoazide (PMA; Biotium Inc., Hayward, CA, USA), a chemical compound that can intercalate the DNA of non-viable (or membrane-damaged) organisms but is excluded from viable bacteria. After light activation, PMA binds covalently to the DNA, preventing its amplification by PCR [3]. After light exposure, unbound PMA is not able to interact further with DNA molecules.

The assay was first optimised using acid-fast bacilli (AFB)-negative sputum samples spiked with dead or live mycobacteria at different concentrations. In brief, live *Mycobacterium fortuitum* cells were added to *N*-acetyl-cysteine decontaminated sputum specimens negative for AFB by smear microscopy at a final concentration of 10^6 bacteria·mL^{-1}. An aliquot of this laboratory-made sample was treated to heat kill the *M. fortuitum* cells. PMA stock solution was prepared and stored at -20°C and protected from light exposure, until use, as recommended by the manufacturers. PMA was added as a pre-treatment at a final concentration of 500 μM and incubated for 30 min at 4°C in the dark, followed by light exposure to blue light-emitting diode (LED)-active light (GenIUL, Terrassa, Spain) for 15 min at room temperature. DNA was then extracted using a standard phenol chloroform procedure. As a control to evaluate the efficacy of the light exposure step, an aliquot of naked DNA extracted from an *M. fortuitum* culture was treated with 500 μM PMA previously exposed to the LED light for 15 min. The commercial line-probe assay (GenoType® Mycobacterium CM; Hain Lifescience, Nehren, Germany) was then performed in order to identify clinically relevant mycobacterial species [4]. Samples containing live *M. fortuitum* showed a normal hybridisation profile on a nitrocellulose strip, whereas samples treated to kill bacteria did not show any amplification, except for the internal control (data not shown). Since naked DNA treated with light-inactivated PMA showed a normal hybridisation profile, the light exposure step was efficient and the PCR was not further inhibited by residual PMA.

Having optimised the protocol for the inactivation of DNA derived from dead bacteria, we adapted it to the Xpert® MTB/RIF automated assay (Cepheid, Sunnyvale, CA, USA). The real-time PCR performed by the Xpert® MTB/RIF provides threshold cycles (Ct) that can be used to calculate the difference in amplification yield between samples with and without PMA pre-treatment (ΔCt): a low ΔCt indicates the presence of amplifiable DNA from live bacteria, whereas a high ΔCt indicates that target DNA in the sample originated from dead or damaged bacteria and, therefore, PMA pre-treatment significantly affects its amplification. To calculate the ΔCt value we considered the mean between the Ct values provided for each probe included in the Xpert® MTB/RIF test (A to E) [5].

We tested the PMA protocol using the Xpert® MTB/RIF assay on a Ct calibration curve of AFB-negative clinical specimens with heat-killed/live mycobacteria added in different ratios. High dead–live ratios resulted in a maximal difference in ΔCt values between heat-treated and live portions with PMA, whereas samples containing a similar percentage of killed and live bacteria could not be differentiated from one another by the use of PMA