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# Drug-resistance - a challenge for tuberculosis control in the European Union and European Economic Area

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Worldwide resistance to antituberculosis drugs is jeopardising the control and eventually the elimination of tuberculosis (TB). Patients who are infected by *Mycobacterium tuberculosis* resistant to antituberculosis drugs, especially to both isoniazid and rifampicin and thus having multidrug-resistant (MDR) TB, need more complicated treatment that lasts longer and consists of a large number of drugs and frequently leads to side effects. In the European Union and European Economic Area (EU/EEA), treatment outcome results for all types of drug resistance were below the target of 85% set by the European Centre for Disease Prevention and Control (ECDC), for new pulmonary culture-positive cases and with 34% the reported treatment success rate of MDR-TB cases was substantially below the target of 70% [1, 2]. Of TB cases diagnosed with MDR-TB in 2009 in the EU/EEA, almost two-thirds died during treatment, failed their treatment or defaulted from treatment. This failure to ensure adequate MDR-TB treatment not only puts patients' lives at risk but also paves the way for XDR TB, and further transmission of the disease.

Analysis of the worldwide data for TB from 2007 to 2010, showed proportions of MDR-TB of 3.4% in new TB cases and 19.8% in those previously treated [3]. The proportions of TB cases with MDR-TB are similar in the EU/EEA with 2.6% of the new culture-confirmed TB cases diagnosed with MDR-TB and 18.8% of previously treated culture-confirmed TB cases [2]. In this issue of *Eurosurveillance* and in the 13 March issue, studies on surveillance of drug-resistant TB, including molecular surveillance, are published. These studies show that drug-resistant TB occurs in diverse settings in Europe and that there is ongoing transmission of drug-resistant TB also in countries with a low TB incidence.

Most studies on TB and drug resistance focus on multidrug resistance. Using data submitted to the EU/EEA TB surveillance system, ECDC performed an analysis of all types of drug-resistant TB notified in the EU/EEA in the period from 2007 to 2012 [4]. In this six-year period, the proportion of TB cases with different drug resistance patterns has been stable with about 90% of

the new laboratory-confirmed TB cases pan-susceptible, 6% monodrug-resistant, 2% polydrug-resistant, 2% MDR-TB, excluding extensively drug-resistant TB (XDR-TB), and 0.2% XDR-TB.

The fact that drug resistance does not seem to increase in the EU/EEA is good news in principle. However, with proportions of MDR-TB ranging from 20.6 to 46.7% in laboratory confirmed cases in neighbouring countries [2], EU/EEA countries need to be vigilant and prepared for tackling an increase in drug resistance. This is supported by the results from the study by Jenkins et al. in this issue, who analysed routinely collected surveillance data in Georgia [5]. They identified between January 2009 and June 2011, 1,795 incident MDR-TB cases confirmed through DST leading to a nationwide MDR-TB incidence of 16.2 notified cases per 100,000 population with considerable geographic variation from 0.0 to 5.0 for new MDR-TB cases and from 0.0 to 18.9 for previously treated cases. In prisons incidence was as high as 837 per 100,000.

A study by C Ruesen et al. from the Netherlands also evaluated all types of TB drug resistance [6]. In contrast to the overall findings for the EU/EEA, they revealed that antituberculosis drug resistance increased in the Netherlands since 1993 in patients born in the Netherlands, and since 2005, in foreign-born patients. Furthermore, only a small fraction (8%) of the identified cases seemed to have acquired drug resistance, while most (92%) were considered to be infected by a resistant strain. Most transmission of drug resistant TB, over 60%, occurred before 1993 or abroad. In only 9% of the cases the transmission definitely took place in the Netherlands.

A molecular surveillance study in Switzerland including all 49 MDR-TB strains identified in the period 2006 to 2012, showed that 12 strains were grouped into six clusters [7]. In-country transmission was likely in four clusters. Most other strains were obtained from MDR-TB cases of foreign origin and were likely imported to Switzerland.

Also in the current issue of *Eurosurveillance*, transmission of MDR-/ XDR-TB strains across the borders of EU/EEA countries is presented based on data from a ECDC initiated molecular surveillance project on MDR-/XDR-TB in the EU [8]. Cross-border transmission of MDR-TB was defined as at least two strains with identical 24-locus variable number of tandem repeat (VNTR) typing patterns, where the cases are in at least two different EU/EEA countries. Almost half (45%) of the strains collected proved to be part of international European clusters and of these, 60% were part of a single, large European cluster. Real transmission of MDR-/XDR-TB, however, is likely to be higher since the project only covered 12% of the total number of MDR-/XDR-TB cases notified in the in the period 2003-2011.

The above studies show that MDR-TB is both imported into and transmitted within the EU/EEA. To prevent transmission of MDR-TB early diagnosis, including use of rapid tests for diagnosis of drug resistance, infection control, and contact investigation are essential. Furthermore, transmission of TB is prevented by timely and adequate treatment which shortens the infectious period. According to a recent survey in Europe, tracing of contacts has been implemented in all participating EU/EEA countries. While this is very positive, the survey also showed that rapid diagnostic tests are not available in all countries, nor is TB infection control implemented everywhere [9]. This demonstrates that there is room for more rigorous implementation of measures to avoid transmission of TB and MDR-TB in the EU/EEA.

To prevent importation of TB and MDR-TB, migrant TB screening seems to be an attractive intervention. Eleven EU countries have implemented migrant screening [10]. All respective screening programmes reported the use of chest radiography to screen for active disease. The yield of this screening is however, low [10,11]. Also, use of chest radiography screening will not identify individuals infected with TB but not yet symptomatic. Therefore, it is of utmost importance that healthcare systems are accessible for migrants and that healthcare is affordable for them so that they can search for care as soon as symptoms of active TB develop.

MDR-TB can only be diagnosed when *M. tuberculosis* bacilli are present in sputum or other samples. Since TB in children is often paucibacillar, laboratory confirmation can be difficult [12]. Of all paediatric cases reported to the EU/EEA TB surveillance system between 2000 and 2009, only 16.9% were confirmed by culture [13]. Sanchini et al. in this issue, [14] analysed routine laboratory data of several national TB reference laboratories in the EU to assess laboratory procedures for diagnosis of paediatric TB. All laboratories receiving primary samples performed the whole range of diagnostic tests, i.e. smear microscopy, molecular identification, culture, and first- and second-line drug susceptibility testing. In the period 2007-2011, 156 of 5,156 (3.0%) samples from children tested positive for

*Mycobacterium tuberculosis* (complex) and of these 10 (6.4%) showed multidrug resistance. This is higher than the 4.7% MDR TB reported for all TB cases in 2012 [4]. Thus MDR-TB cannot be neglected in children and intensive efforts should be applied to collect samples from children for laboratory confirmation of TB. Since children are often not able to produce sputum samples spontaneously, gastric aspiration and sputum induction may be applied instead [15]. Also, even though more invasive, fine-needle aspiration biopsy has proved to be useful for collecting samples in children with a peripheral lymph-node mass [16]. In addition to facilitating the diagnosis of TB and drug resistance, strains obtained from positive cultures can also be subjected to molecular typing and provide valuable information about transmission of TB such as demonstrated in several studies in this issue [6,7,8].

Since unsuccessful treatment outcomes are reported frequently within the EU/EEA, especially for MDR-TB cases, those involved in treatment and care of MDR-TB patients should do everything possible to arrive at better treatment outcomes [2]. To assist healthcare workers with the management of drug-resistant TB cases several tools were developed. First of all, the 'European Union Standards for Tuberculosis Care', these include standards for treatment of TBs, including MDR-TB [17,18]. In addition, a consensus statement on management of patients with M/XDR-TB in Europe has been developed recently [19]. A specific tool to assist healthcare workers is the 'Electronic Consilium' which was launched to provide scientifically sound and evidence-based clinical consultation for drug-resistant TB and other difficult-to-treat TB cases [20]. Between the start of the consilium in September 2012 and July 2013, the platform has supported the clinical management of ten TB patients [21]. We hereby call on all healthcare workers to make use of the consilium and other tools to guarantee proper management of patients with drug resistant TB and to cure them

In conclusion, early diagnosis of all cases with MDR-TB, adequate treatment of MDR-TB patients and implementation of infection control measures to prevent further transmission, are crucial to overcome challenges posed by MDR-TB. Realising the full implementation of these prevention and control measures should be a priority for policy makers and healthcare workers engaged in controlling and eliminating TB and MDR-TB.

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# Molecular surveillance of multi- and extensively drug-resistant tuberculosis transmission in the European Union from 2003 to 2011

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The European Centre for Disease Prevention and Control (ECDC) initiated a project on the molecular surveillance of multi- and extensively drug-resistant tuberculosis (MDR-/XDR-TB) transmission in the European Union (EU) in the period from 2009 to 2011. In total, 2,092 variable number of tandem repeat (VNTR) patterns of MDR-/XDR-TB *Mycobacterium tuberculosis* isolates were collected, originating from 24 different countries in the period 2003 to 2011. Of the collected VNTR patterns, 45% (n=941) could be assigned to one of the 79 European multiple-country molecular fingerprint clusters and 50% of those (n=470) belonged to one extremely large cluster caused by Beijing strains of one genotype. We conclude that international transmission of MDR-/XDR-TB plays an important role in the EU, especially in the eastern part, and is significantly related to the spread of one strain or clone of the Beijing genotype. Implementation of international cluster investigation in EU countries should reveal underlying factors of transmission, and show how TB control can be improved regarding case finding, contact tracing, infection control and treatment in order to prevent further spread of MDR-/XDR-TB in the EU.

## Introduction

Molecular surveillance of multi- and extensively drug-resistant tuberculosis (MDR-/XDR-TB) in the European Union (EU) on basis of *IS6110* restriction fragment length polymorphism (RFLP) typing detected large molecular clusters of MDR-/XDR-TB cases across EU countries in the period 2003 to 2007 [1]. It also identified possible transmission patterns and risk factors for MDR-TB and XDR-TB, such as country of origin and infection with the Beijing genotype [2]. Following up on these findings, the European Centre for Disease Prevention and Control (ECDC) initiated a molecular surveillance project on MDR-/XDR-TB in the EU from

2009 to 2012 which was built on the existing TB network previously funded by the European Commission. This new project, carried out by the National Institute for Public Health and the Environment (RIVM) on behalf of the ECDC, aimed at achieving a higher coverage by expanding molecular typing to countries in the EU where this was not yet the practice. For this purpose, the 24-locus mycobacterial interspersed repetitive unit variable number of tandem repeat (MIRU-VNTR) typing method was selected as the main DNA fingerprinting methodology [3]. This method has become the international gold standard for typing of *Mycobacterium tuberculosis* isolates and offers important advantages over *IS6110* RFLP typing, while its discriminatory power equals that of *IS6110* [3,4]. Firstly, VNTR typing is easier to perform than RFLP typing and can be implemented more efficiently in countries that do not yet perform molecular typing. Secondly, it is based on DNA amplification, which abolishes the need for culture of *M. tuberculosis* and has a shorter laboratory turnaround time. Moreover, this approach uses low quantities of DNA and allows exchange of (non-viable) mycobacterial culture material by regular mail. Finally, the results of VNTR typing are in a simple format, which facilitates efficient exchange of typing information and inter-laboratory comparison. In principle, this introduces more real-time typing and rapid feedback on molecular clustering to identify newly emerging MDR-/XDR-TB strains.

This paper describes the major findings of the ECDC/RIVM project regarding the detection of international clusters, the molecular typing coverage of MDR-/XDR-TB cases, the conclusions drawn from molecular analysis and recommendations for the future development of molecular surveillance of MDR-/XDR-TB in the EU.



**TABLE 1**

Culture-confirmed multi- and extensively drug-resistant tuberculosis cases reported to the TESSy system, and coverage in the molecular surveillance project, by country, 2003–2011 (n=16,858)

Country of isolation	Year of isolation									Total reported to ECDC 2003–11	Total with molecular surveillance data 2003–11	Coverage
	2003	2004	2005	2006	2007	2008	2009	2010	2011			
Austria	12	19	13	10	9	15	22	15	19	134	NR	NA
Belgium	9	12	11	18	14	21	10	19	15	129	30	23%
Bulgaria	44	47	47	53	76	31	43	56	55	452	141	31%
Croatia	8	3	7	3	3	4	7	NR	2	37	56	151% <sup>a</sup>
Cyprus	0	0	1	0	3	1	4	0	1	10	1	10%
Czech Republic	2	6	13	12	8	11	8	9	7	76	37	49%
Denmark	0	0	5	3	2	0	2	2	3	17	15	88%
Estonia	106	90	78	55	82	73	85	63	78	710	557	78%
Finland	3	0	2	2	2	1	6	6	5	27	20	74%
France	25	26	24	30	20	27	30	23	40	245	87	36%
Germany	91	98	103	82	66	49	62	48	56	655	62	9%
United Kingdom	52	47	40	51	56	53	60	60	81	500	120	24%
Greece	22	16	12	13	14	0	14	0	5	96	48	50%
Hungary	20	11	17	14	12	13	18	19	7	131	36	27%
Ireland	1	2	3	4	7	2	1	2	3	25	18	72%
Italy	42	24	22	28	56	71	82	87	81	493	248	50%
Latvia	174	195	161	142	99	128	131	87	95	1,212	30	2%
Liechtenstein	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	NR	NA
Lithuania	312	318	338	332	314	276	322	310	296	2,818	73	3%
Luxembourg	1	1	0	0	0	0	0	0	2	4	NR	NA
Norway	3	4	3	3	3	4	8	8	4	40	28	70%
Poland	92	51	46	32	22	19	21	30	41	354	NR	NA
Portugal	23	35	31	22	34	32	24	19	22	242	NR	NA
Romania	585	810	849	673	673	792	624	502	530	6,038	NR	NA
Slovakia	6	1	8	7	7	4	1	1	3	38	13	34%
Slovenia	1	0	1	1	0	2	1	0	0	6	6	100%
Spain	47	59	80	50	59	76	56	49	41	517	242	47%
Sweden	7	6	4	3	15	11	13	18	17	94	75	80%
The Netherlands	8	3	3	5	3	13	20	11	15	81	92	114% <sup>a</sup>
Turkey	0	0	191	249	240	263	222	250	262	1,677	20	1%
<b>Total</b>										<b>16,858</b>	<b>2,055</b>	<b>12%</b>

NA: not applicable; NR: not reported; ECDC: European Centre for Disease Prevention and Control; TESSy: The European Surveillance System at ECDC.

<sup>a</sup> More than 100% coverage is the result of incomplete culture data collection by the ECDC.

## Methods

### Project design

Molecular typing data of MDR-/XDR-TB cases from EU countries were collected in the period from 2009 to 2011 by the RIVM in Bilthoven, the Netherlands. Furthermore, retrospective typing of isolates collected from patients in the period from 2003 to 2008 and real-time typing of isolates collected from patients from 2009 to the end of 2011 were included. The RIVM reported clustering of MDR-/XDR-TB cases to the ECDC on a regular basis. In addition, the implementation,

standardisation and quality control of VNTR typing in all participating countries was facilitated by ad hoc email contact, on-site training, by project meetings and workshops, and also by the introduction of a proficiency testing programme for VNTR typing [5]. The collection of samples did not follow a rational selection but was driven by the specific situation in the different participating countries.

### Participants in the project

This molecular surveillance project was designed for all EU, European Economic Area (EEA), and EU

candidate countries. The countries with national reference laboratories participating in the project were: Austria, Belgium, Bulgaria, Croatia, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Liechtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Turkey and the United Kingdom.

### MIRU-VNTR typing

The standard for typing *M. tuberculosis* complex strains was the method described by Supply et al. in 2006 [3]. The RIVM offered MIRU-VNTR typing to the countries that were not able to perform the technique locally or were not performing it for other reasons. Specifically, the RIVM performed VNTR typing for Cyprus, the Czech Republic, Estonia, Greece, Hungary, Latvia, Norway, Slovakia and Spain, and partial typing for Finland and Lithuania.

### Drug susceptibility testing

Phenotypic drug susceptibility testing (DST) was performed by the TB reference laboratories participating in the project. All *M. tuberculosis* isolates were tested at least for resistance to the first-line antibiotics rifampicin and isoniazid, and part of the strains were also tested for resistance against second-line antibiotics such as fluoroquinolones and the injectable drugs (capreomycin and aminoglycosides), according to national guidelines for DST. All participating laboratories were members of the European Reference Laboratory Network (ERLN)-TB and had their own national accreditation.

### Molecular assessment of susceptibility by MTBDRplus assay

We selected for molecular assessment strains that belonged to the largest European MDR-TB cluster, with a view of including a wide spread of country and year of isolation. For selected strains, the GenoType MTBDRplus reverse line blot method (HAIN Lifescience, Nehren, Germany) [6] was applied to detect mutations in the *rpoB* gene associated with rifampicin resistance and mutations in the *katG* gene and the *inhA* gene associated with isoniazid resistance.

### Coverage

Based on the tested samples from the period 2003 to 2011, we defined the coverage of molecular fingerprinting of MDR-/XDR-TB as the percentage of MDR-/XDR-TB isolates included in the molecular surveillance project among the total number of MDR-/XDR-TB cases officially reported to the ECDC for the same period. The ECDC published the surveillance results in The European Surveillance System (TESSy) and in the annual surveillance reports.

### Clustering

A European cluster was defined as two or more MDR-/XDR-TB strains with identical 24-locus VNTR typing patterns, isolated in at least two different countries.

Results for 15-locus VNTR typing and VNTR patterns for which one or more loci were missing were also included in the cluster analysis.

### Beijing genotype identification

The Beijing genotype was identified by the specific Beijing branch of the dendrogram with a similarity percentage of 24-locus VNTR typing of at least 60%. The Beijing branch was determined by 656 isolates confirmed as the Beijing genotype based on spoligotyping. The non-Beijing branches were confirmed as such by spoligotyping of 201 isolates.

## Results

### Coverage

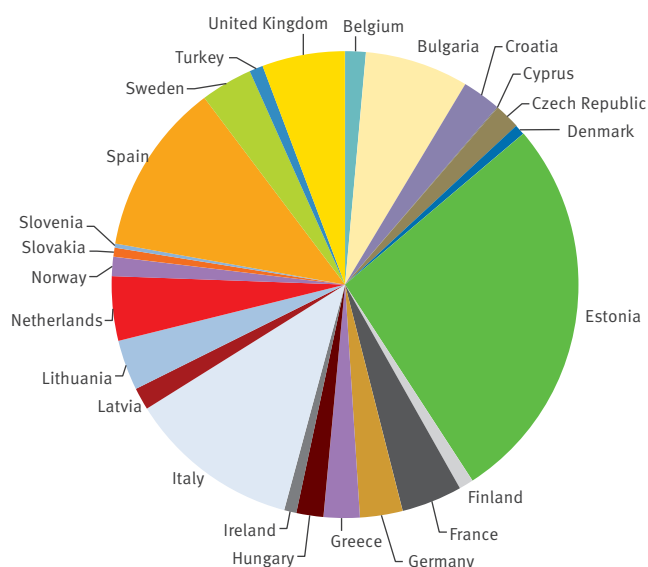
The countries participating in the project reported 16,858 MDR-/XDR-TB cases to the ECDC for the period 2003 to 2011. The total number of MDR-/XDR-TB isolates collected in that period for which VNTR typing data were available amounted to 2,055. Therefore, the coverage of the molecular surveillance for the period 2003 to 2011 was 12%. Six countries reported no molecular typing results at all; excluding these countries, the coverage was 20%. The coverage differed significantly by country and year (Table 1).

### Typing

We collected 2,092 VNTR patterns, originating from 2,055 MDR-/XDR-TB patients sampled between 2003 and 2011 in 24 different countries (Figure 1). There were more VNTR patterns than isolates because double alleles were detected in the VNTR patterns of 37

**FIGURE 1**

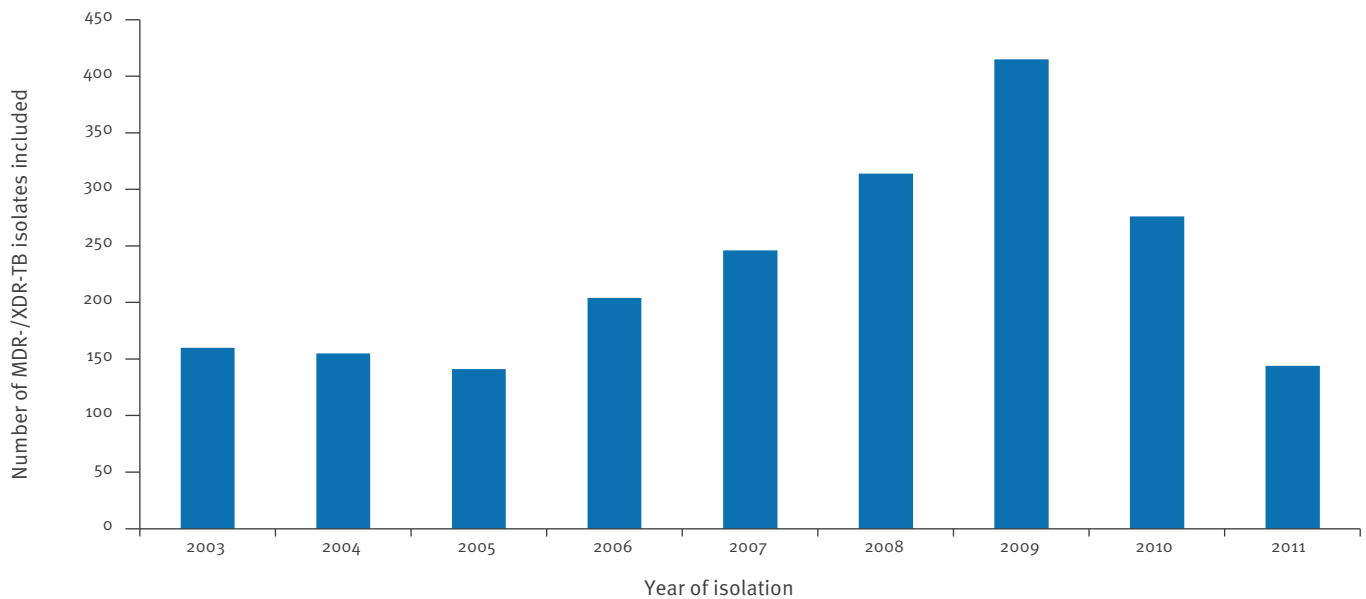
Number of VNTR patterns of multi- and extensively drug-resistant *Mycobacterium tuberculosis* isolates included in the molecular surveillance project, by country of isolation, sampled 2003–2011 (n=2,092)



VNTR: variable number of tandem repeat.

**FIGURE 2**

*Mycobacterium tuberculosis* isolates included in the molecular surveillance project, by year of isolation, 2003–2011 (n=2,055)



MDR-/XDR-TB: multi- and extensively drug-resistant tuberculosis.

isolates that were included in the project database as separate patterns. For 53% (n=1,093) of the included isolates, the typing results were produced by the reference laboratory of the country of isolation, and for 47% (n=962) the molecular typing was performed at the RIVM.

The number of isolates included per year is depicted in Figure 2; 2009 was the year with the highest number of isolates included (n=415). The sex was known for 69% (n=1,428) of the cases whose isolates were typed: 70% (n=999) of the MDR-/XDR-TB cases were male and 30% (n=429) female. The age at the time of TB diagnosis was available for 68% (n=1,402) of the MDR-/XDR-TB cases included in this study: their mean age was 40 years (range: 1–88 years).

### Clustering

Comparison of the 2,092 VNTR patterns included in the project resulted in the detection of 79 European clusters. The cluster sizes varied from two to 470 cases per cluster (Figure 3). In total, 45% (n=941) of all the collected VNTR patterns were part of a European cluster. The geographic composition of these molecular clusters ranged from two to 17 countries.

For 73% (n=691) of the European clustered cases, the country of origin of the patient was known. In total 73% (n=505) of these patients were resident in the country of isolation and 27% (n=186) originated from abroad. Excluding all clustered cases from Estonia (n=490 for which the country of origin was known) because of the overrepresentation of samples from Estonia, the

distribution was 44% (n=89) and 56% (n=112), respectively, for the 201 samples for which country of origin was known.

The percentage of samples assigned to a European MDR-/XDR-TB cluster, for the countries which submitted at least 10 isolates to the project database, varied from 0 to 87% by country. Clustering on national level was also analysed in this study and varied from 0 to 92% by country (Figure 4).

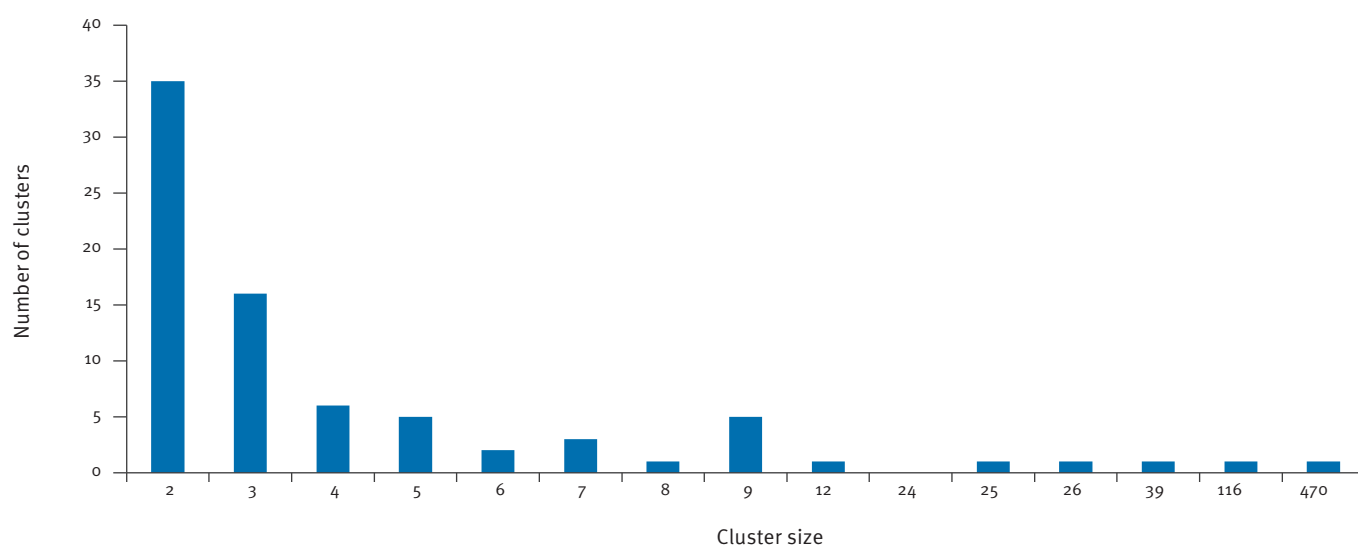
A number of the VNTR typing patterns (n=465; 22%) did not cover all of the 24 loci due to technical problems or because these loci were not tested in the participating laboratories. In total 60 samples with incomplete VNTR patterns were part of molecular clusters (among them 32 samples of the Beijing genotype): 48% (n=29) of the samples with incomplete VNTR patterns were part of 22 European clusters, while 52% (n=31) of them belonged to European clusters which had already been defined on the basis of 24-locus VNTR results from at least two other samples from two different countries.

Of all clustered isolates included in the project database, 60% (n=470) were part of one large VNTR typing cluster (Figure 3; Table 2). This molecular cluster, comprising a VNTR pattern with a Beijing genotype signature, has so far been detected in 17 EU countries. The majority of cases that belonged to this cluster were detected in the Baltic States, mainly in Estonia (Figure 5). Because of the high coverage of reported cases in Estonia, 98% for the period 2003 to 2009, the growth dynamics of this largest molecular cluster are depicted



**FIGURE 3**

Size of European clusters of multi- and extensively drug-resistant tuberculosis cases detected in the molecular surveillance project, 2003–2011 (n=79 clusters)



in Figure 6. In 2009, 72 isolates in the cluster originated from Estonia; in the following years, this number decreased to 42–55 isolates per year.

For a selection of 48 (10%) isolates in the largest molecular cluster, isolated in different countries and years, we determined the mutations underlying the resistance mechanism. All but one of the tested MDR-/XDR-TB isolates in the VNTR cluster with Beijing genotype revealed the same combination of mutations associated with rifampicin and isoniazid resistance: *rpoB* S531L and *katG* S315T. One exceptional MDR-/XDR-TB isolate harboured the *rpoB* H526Y and *katG*

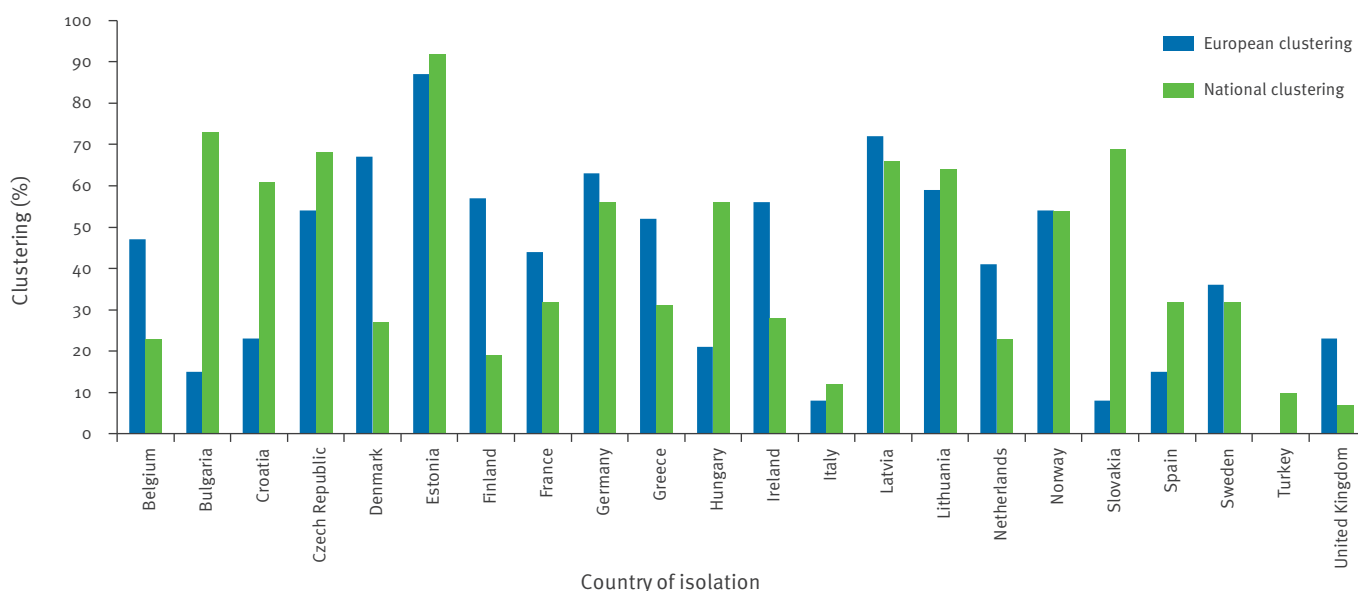
S315T mutations. For 39 of these 48 strains, the resistance to fluoroquinolones and the injectable drugs was tested phenotypically: 12 were resistant to both, five only to fluoroquinolones, 12 only to injectable drugs, and 10 showed no resistance.

### Characteristics of clustered MDR-/XDR-TB cases

Sex and age did not differ between clustered and non-clustered cases. The overall mean age was 40 years (range: 1–88 years). The percentage of VNTR patterns who were part of a European cluster was 54% (n=548)

**FIGURE 4**

Percentage of cases in European (n=941) and national (n=1,086) clusters of multi- and extensively drug-resistant tuberculosis, by country, molecular surveillance project, 2003–2011



**TABLE 2**

24-locus VNTR pattern of the largest multi- and extensively drug-resistant tuberculosis cluster with a Beijing genotype signature detected in the European Union, 2003–2011 (n=470 isolates)

Genome position number	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	154	2531	4348	2059	2687	3007	2347	2461	3171
Number of tandem repeats	2	7	3	3	3	5	4	4	4	4	3	2	6	5	7	2	5	3	2	1	3	4	2	3

VNTR: variable number of tandem repeat.

for isolates from male cases and 49% (n=213) for isolates from female cases.

Forty-four per cent (n=920) of the analysed VNTR patterns of MDR-/XDR-TB isolates were assigned to the Beijing genotype with a similarity of at least 74% on the basis of 24-locus VNTR typing. For 71% (n=656) of the 920 isolates, the Beijing genotype was confirmed by RFLP typing and/or spoligotyping and a non-Beijing genotype was confirmed for 17% (n=201) of the 1,173 strains identified as non-Beijing.

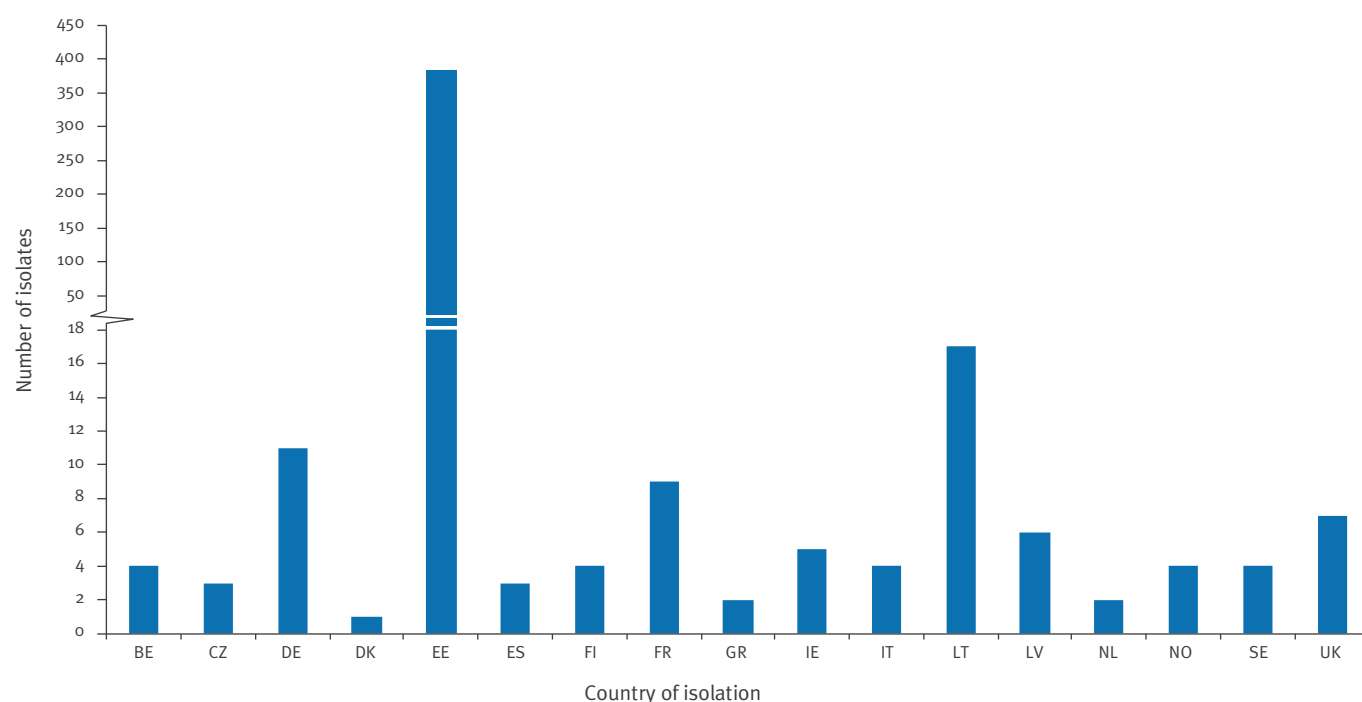
In total, 77% (n=726) of the clustered cases were caused by Beijing genotype strains with 37 different VNTR patterns (the two largest molecular clusters were caused by Beijing genotype strains). Among non-clustered

cases, 17% (n=194) were caused by Beijing strains ( $p<0.05$ ). The mean age for MDR-/XDR-TB cases caused by Beijing genotype strains was not different from that of non-Beijing MDR-/XDR-TB cases: 41.9 vs 39.5 years. In relation to the sex distribution, the Beijing genotype was more often detected in male than in female patients: 53% (n=539) vs 47% (n=206).

The susceptibility of the *M. tuberculosis* strains to second-line drugs was known for 53% (n=1,080) of the isolates. Twelve per cent (n=132) of them were XDR-TB, and 135 VNTR patterns were found for them. There were significantly more men than women among XDR-TB patients: 69% (n=91) vs 23% (n=31) ( $p<0.05$ ). XDR-TB was significantly more often detected in MDR-TB strains of the Beijing genotype than in MDR-TB

**FIGURE 5**

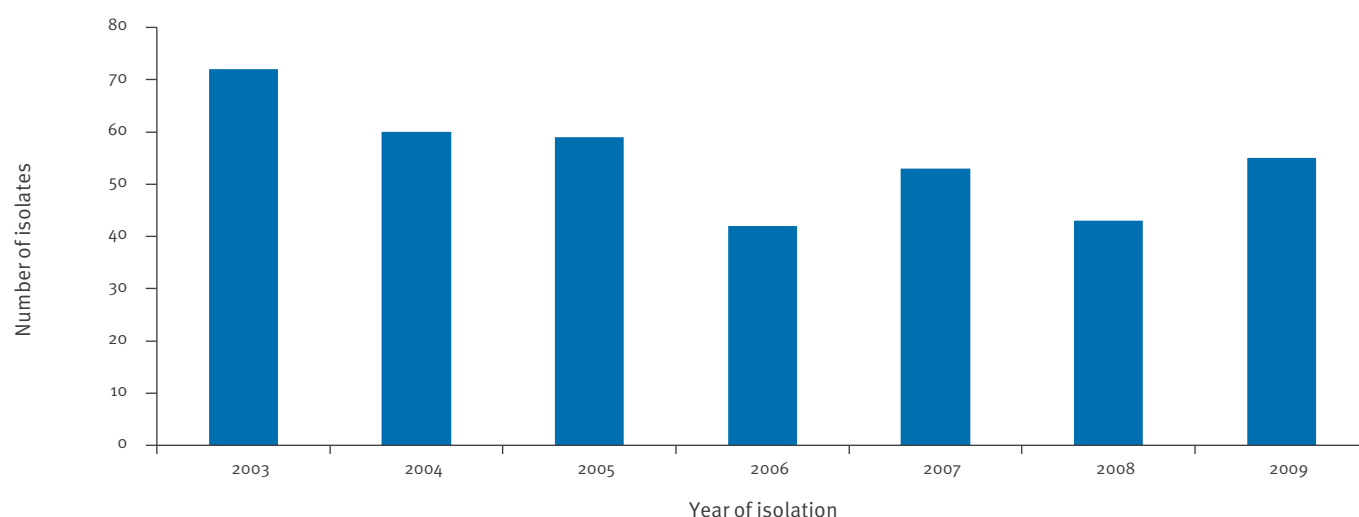
Geographical distribution of cases in the largest European multi- and extensively drug-resistant tuberculosis cluster 2003–2011 (n=470)



BE: Belgium; CZ: Czech Republic; DE: Germany; DK: Denmark; EE: Estonia; ES: Spain; FI: Finland; FR: France; GR: Greece; IE: Ireland; IT: Italy; LT: Lithuania; LV: Latvia; NL: the Netherlands; NO: Norway; SE: Sweden; UK: United Kingdom.

**FIGURE 6**

Multi- and extensively drug-resistant *Mycobacterium tuberculosis* isolates belonging to the largest European cluster, by year, Estonia, 2003–2009 (n=384)



strains of non-Beijing genotypes: 86% (n=116) vs 14% (n=19) ( $p < 0.01$ ). In addition, 78% (n=105) of the XDR-TB VNTR patterns belonged to eight international clusters; six of these clusters were determined as the Beijing genotype.

## Discussion

Almost half of the VNTR patterns collected in this molecular surveillance study of MDR/XDR *M. tuberculosis* was assigned to international European clusters, and 60% of these were part of a single, large European cluster. This molecular cluster, associated with the spread of a Beijing genotype strain, has so far been detected in 17 European countries. It was previously described in the EU by RFLP typing and notified for the first time in 2003 [1,2]. The RFLP typing results were available for 63% (n=125) of the isolates obtained from the largest VNTR cluster in 2003 to 2005. This confirmed the clustering of these cases on the basis of both RFLP and VNTR typing. Overall, the Beijing genotype was significantly associated with clustering, and therefore with possible (international) transmission and spread.

The high proportion of molecular clustering (45%) in the EU suggests that MDR/XDR-TB cases may be transmitted and not acquired. The lack of coverage and the wide variation in the number and time period of collected samples submitted by the participating countries, however, reduce the representativeness of this observation. Furthermore, a high proportion of the European clustered cases (73%) were patients originating from the country of isolation rather than immigrants. Even when excluding all Estonian isolates, the percentage was still 44%. This confirms that MDR/XDR-TB transmission was taking place and that not all detected molecular clusters were a result of human migration.

The high percentage of European and national clustering, especially in Estonia (87% and 92%) and Latvia (72% and 66%), indicates that transmission has been ongoing in this region for a prolonged period [7], and this calls into question the infection control practices and the quality of treatment. In contrast, the low percentage of clustering in Italy (8%) and Spain (15%) indicates that the MDR-TB problem in these regions is mainly due to TB imported by immigration from countries not participating in the project, as suggested earlier [8,9]. In addition, countries with a higher percentage of European clustering compared to the percentage of national clustering, e.g. the Netherlands (41% vs 23%) and Finland (57% vs 19%), are examples of importation of MDR/XDR-TB from European countries and a health system that prevents national transmission.

XDR-TB was detected in 12% (n=132) of the *M. tuberculosis* isolates for which second-line drug susceptibility data was available. This is slightly higher than described earlier for the MDR-TB cases examined in the period 2006 to 2009 [2]. The Beijing genotype is associated with multidrug resistance in many settings [10]. In this European surveillance project, the Beijing genotype was significantly associated with XDR-TB, in contrast to strains of non-Beijing genotypes: respectively 86% (n=116) and 14% (n=19). The association of the Beijing genotype with resistance has been studied extensively; potential underlying mechanisms include a higher mutation frequency of the *rpoB* gene in strains of the Beijing genotype, resulting in a higher ability to withstand rifampicin exposure [11].

The most important limitation of our study is the poor coverage and thus the possible selection bias; the percentage of MDR/XDR *M. tuberculosis* isolates that were actually submitted by the participating countries in the period from 2003 to 2011 ranged from 0% to more than 100%. Limited coverage also affected the timeliness

of delivery of data. Several countries, including a few large ones, reported limited data, although it was agreed in the project to send real-time typing results. The effect of this limitation is a possible underestimation of international transmission of MDR-/XDR-TB in the EU. An important implication of our study is that especially in western EU countries, the percentage of clustered MDR-/XDR-TB cases is low. This implies that resistance was either acquired in the patient in the country where the strain was isolated, or a consequence of sequential import of unrelated cases from endemic regions.

In contrast, in the eastern EU countries and especially the Baltic States, a large proportion of MDR-/XDR-TB isolates belonged to molecular clusters. Moreover, one large molecular cluster of 470 cases was caused by Beijing strains with identical 24-locus VNTR typing patterns. This implies major and ongoing transmission of an easily transmissible and virulent strain or clone. Forty-seven of the 48 tested isolates in the largest molecular cluster had the same combination of *rpoB* S531L and *katG* S315T mutations, associated with rifampicin and isoniazid resistance. There is bacteriological and epidemiological data demonstrating that these mutations result in the lowest loss of fitness in isoniazid- and rifampicin-resistant bacteria [12,13]. Resistance to second-line drugs was high variable. The largest international cluster may therefore be caused by one successful MDR-/XDR-TB strain that is responsible for many transmissions, with resistance to second-line drugs developing further in the affected patients. Alternatively, we may be observing the spread of genetically highly similar strains of the Beijing genotype. By whole-genome sequencing, the true percentage of similarity can be determined, and this will help to answer this question.

Another important limitation in this study was the lack of epidemiological data to confirm chains of human transmission. Although the typing data are highly suggestive of spread of successful strains, this still needs to be confirmed.

For this project, we selected VNTR typing as the standard method. This technique was previously shown to be highly reproducible, both within [14] and between laboratories [15]. However, the participants of the ECDC/RIVM project used a large variation in protocols and methodologies and had different levels of experience in performing VNTR typing. Therefore, we performed two proficiency studies; initial results were disappointing regarding both the intra- and inter-laboratory reproducibility [5]. Although several suggestions for improvements were communicated to participants, this lack in quality may still have influenced the results of the current study, leading to an underestimation of clustering cases. After implementation of several improvements in the methodology and a higher degree of standardisation, the second international proficiency study in 2010 on VNTR typing yielded much better results [16].

In conclusion, large-scale international transmission of MDR-/XDR-TB occurs within the EU and demands increased surveillance and public health action. The *M. tuberculosis* strains with Beijing genotype are large drivers of this international transmission and are associated with the emergence and spread of XDR-TB.

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## Conflict of interest

None declared.

## Authors' contributions

The project participants all contributed significantly to the results of this study.

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# Extent and origin of resistance to antituberculosis drugs in the Netherlands, 1993 to 2011

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The elimination of tuberculosis (TB) is threatened by an apparent increase in the level of resistance in *Mycobacterium tuberculosis*. In the Netherlands, where the majority of TB patients are migrants, resistance may also be increasing. We conducted a retrospective study, using 18,294 *M. tuberculosis* isolates from TB cases notified between 1993 and 2011. We investigated the trends in antituberculosis drug resistance, focusing on the country of birth of the patients and whether resistance had developed during treatment or was the result of transmission of resistant *M. tuberculosis* strains. For both scenarios, we determined whether this had happened in or outside the Netherlands. Antituberculosis drug resistance was found in 13% of all cases analysed and showed an increasing trend among patients who had been born in the Netherlands ( $p < 0.001$ ) and a decreasing trend among foreign-born ( $p = 0.02$ ) over the study period. Since 2005, the proportion of *M. tuberculosis* resistant strains among all strains tested has increased in both groups ( $p = 0.03$  and  $p = 0.01$ , respectively). Overall, we found a significantly increasing trend when excluding streptomycin resistance ( $p < 0.001$ ). The trend was most markedly increased for isoniazid resistance ( $p = 0.01$ ). Although resistance was mainly due to transmission of resistant strains, mostly outside the Netherlands or before 1993 (when DNA fingerprinting was not systematically performed), in some cases ( $n = 45$ ), resistance was acquired in the Netherlands. We conclude that antituberculosis drug resistance is increasing in the Netherlands, mostly related to migration from high TB-incidence countries, but also to domestic acquisition.

## Introduction

Resistance to antituberculosis drugs is emerging in several areas worldwide. In eastern Europe and central Asia, hotspots of multidrug-resistant tuberculosis (MDR-TB) are present, with nearly a third of the new and three quarters of previously treated TB cases diagnosed as having MDR-TB in some countries [1]. This is

of great concern, considering the limited drug options to safely and effectively treat these resistant forms of TB. Distinguishing between transmission of a resistant *M. tuberculosis* strain and development of resistance during treatment has important consequences for TB control programmes [2].

The elimination of TB (defined as less than one case per million population) – a World Health Organization (WHO) target for 2050 [3] – is threatened by an apparent increase in multidrug resistance worldwide [1,4,5]. Global trends, however, are hard to interpret as a result of incomplete coverage of surveillance data. In many regions in Sub-Saharan Africa, and also in central and eastern Europe and India, drug resistance surveillance data are lacking, mainly as a result of inadequate laboratory infrastructure [1].

In the Netherlands, a low TB-incidence country with approximately 1,000 new registered TB cases annually and an incidence of 6.0 per 100,000 population in 2011 [6], nationwide surveillance of TB has been in place since 1993. Until recently, all local mycobacteriology laboratories routinely sent their *M. tuberculosis* complex isolates to the WHO-accredited National Reference Laboratory at the National Institute for Public Health and the Environment (RIVM) for identification, drug susceptibility testing (DST) and molecular typing. After 2011, some local laboratories started to screen for resistance against first-line drugs themselves, but when resistance is diagnosed, the results are confirmed at the National Reference Laboratory, where DST is broadened to other drugs. A DNA fingerprint of each *M. tuberculosis* isolate is produced, to guide investigation of epidemiological links between TB cases.

In the mid-1990s, a descriptive study showed that the majority of TB cases with resistant strains in the Netherlands were migrants [7]. Recent increases in the proportion of migrants among TB patients in the Netherlands [6], in the prevalence of drug resistance

in many of the migrants' country of origin [8] as well as changes in the composition of the migrant population might have influenced the resistance situation in the Netherlands over the last couple of years [6,9]. To improve our understanding of the extent and origin of *M. tuberculosis* resistance in the Netherlands, we conducted a retrospective study of all TB cases notified between 1993 and 2011. We investigated the trends in resistance to antituberculosis drugs in this period, in relation to the country of origin of the patients. In addition, we assessed the extent to which drug resistance was due to transmission of resistant strains or was possibly acquired during previous treatment. For both scenarios, we determined whether this had occurred in or outside the Netherlands.

## Methods

### Data sources and study population

Data were obtained from three sources and matched on the basis of postal code, date of birth and sex. The resulting data set consisted of anonymous data. We used data from three sources: firstly, the Netherlands Tuberculosis Register after approval by the registry committee. These data, systematically collected at Municipal Health Services, include information on patient characteristics, treatment history, case finding and treatment outcome. Secondly, data from the National Reference Laboratory were used, which contain information on drug susceptibility and DNA profiles of the bacterial isolates. Between 1993 and 2009, nationwide fingerprinting of *M. tuberculosis* isolates using IS6110 restriction fragment length polymorphism (RFLP) typing was performed at the National Reference Laboratory; after 2009, RFLP typing was replaced by variable number tandem repeat (VNTR) typing [10,11]. Thirdly, we received the results of epidemiological investigation of clustered cases, which is routinely carried out by TB public health nurses in the Netherlands.

All notified *M. tuberculosis* culture-positive cases between 1993 and 2011 were included in the study. Isolates with missing DST results, including those tested in local laboratories, which participate in quality control programmes, were considered susceptible. If patients had multiple isolates, only isolates of *M. tuberculosis* strains with different DNA fingerprints were included in the database: subsequent isolates representing the same *M. tuberculosis* strain were excluded.

### Drug resistance: trends and origin

Standard DST for the following first-line drugs was performed for each isolate: isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide. Susceptibility to the following second-line drugs was only assessed if the isolate was resistant to isoniazid and/or rifampicin: amikacin, capreomycin, ciprofloxacin, clarithromycin, clofazimine, cycloserine, kanamycin, linezolid, moxifloxacin, ofloxacin, prothionamide and rifabutin. Until 2004, the absolute concentration method was the

standard DST method used [12], but this was replaced thereafter by the mycobacteria growth indicator tube (MGIT) assay [13]. Patients were classified according to the DST result as having drug-susceptible isolates if the causative bacteria were sensitive to the first-line drugs tested or as having drug-resistant isolates if resistance to at least one drug was detected.

Trends in resistance were analysed for the study period. Extensively drug-resistant TB was only detected rarely and involved in total three cases in 2009 to 2011 [6]. Drug resistance rates (percentage of resistant isolates among all isolates tested for drug susceptibility) were described for those born in the Netherlands and those who were born abroad.

A distinction was made between primary drug resistance (PDR), i.e. drug resistance in new TB cases and acquired drug resistance (ADR), i.e. drug resistance in previously treated TB cases. For foreign-born ADR patients, we compared the year of entry into the Netherlands with the year of previous TB treatment to assess whether resistance had been acquired in the Netherlands or abroad. ADR patients born in the Netherlands were considered to have acquired resistance in the Netherlands.

### Transmission of drug-resistant TB

For PDR cases, we assessed where transmission of the resistant strain most probably occurred, based on the DNA fingerprinting of *M. tuberculosis* isolates and the subsequent results of cluster investigation.

Clusters were defined as groups of patients having isolates with identical RFLP or VNTR patterns or, if strains had fewer than five IS6110 copies, identical polymorphic GC-rich sequence RFLP patterns [14]. During 2004 to 2008, within the framework of a nationwide evaluation following the introduction of VNTR typing, both RFLP and VNTR typing were performed for all isolates and strains could thus belong to both an RFLP and a VNTR cluster [15]. The agreement in clustering between both methods was about 80%. In order to prevent strains being part of two different clusters, we used the RFLP patterns to cluster isolates from before 2009 and VNTR patterns to cluster strains isolated in 2009 or thereafter. Cases were divided into those whose *M. tuberculosis* strain had a unique DNA fingerprint and those with a clustering fingerprint. The first case in each cluster, based on the diagnosis date, was classified as unique. After matching the Netherlands Tuberculosis Register data with data from the National Reference Laboratory, a number of clusters were broken up as a result of a 15% mismatch, which occurred due to incorrect or incomplete data on identifying variables (e.g. country of birth, sex, postal code) that link the cases. Cases for whom the data could not be matched were excluded from the analysis. This resulted in the formation of 'clusters' with only one case left, which were excluded from further analysis.

**TABLE 1**

Determinants of resistance to at least one antituberculosis drug in patients with culture-positive tuberculosis in the Netherlands, 1993–2011

Characteristic	Isolates with DST results n=14,820	All isolates n=14,959	Resistant isolates n=1,890	Susceptible isolates n=12,930	Crude OR (95% CI)	Adjusted OR <sup>b</sup> (95% CI)
	n (%) <sup>a</sup>	n (%) <sup>a</sup>	n (%) <sup>a</sup>	n (%) <sup>a</sup>		
Male sex	8,857 (60)	8,941 (60)	1,106 (58)	7,751 (60)	0.94 (0.86–1.04)	1.02 (0.88–1.18)
Mean age in years (SD)	41 (20)	41 (20)	33 (15)	42 (20)	0.98 (0.97–0.98) <sup>c</sup>	0.98 (0.97–0.98) <sup>c</sup>
Living in an urban area	5,150 (35)	5,218 (35)	653 (35)	4,497 (35)	0.99 (0.89–1.10)	1.09 (0.94–1.28)
Foreign-born	10,165 (69)	10,257 (69)	1,590 (84)	8,575 (66)	2.69 (2.37–3.06) <sup>c</sup>	1.92 (1.55–2.38) <sup>c</sup>
Median number of years in the Netherlands before diagnosis (IQR) <sup>d</sup>	5 (1–14)	5 (1–14)	3 (1–9)	5 (1–15)	0.97 (0.97–0.98) <sup>c</sup>	0.99 (0.97–1.00)
BCG vaccination <sup>e</sup>	4,124 (50)	4,182 (50)	667 (67)	3,457 (48)	2.20 (1.92–2.53) <sup>c</sup>	1.06 (0.87–1.28)
Belonging to a risk group	9,265 (63)	9,335 (62)	1,390 (74)	7,875 (61)	1.78 (1.60–1.99) <sup>c</sup>	1.15 (0.96–1.37)
Previous TB treatment <sup>e</sup>	533 (4)	539 (4)	122 (8)	411 (4)	2.19 (1.78–2.70) <sup>c</sup>	2.31 (1.71–3.13) <sup>c</sup>
Median number of years between diagnosis and previous treatment (IQR) <sup>f</sup>	21 (4–50)	21 (5–50)	5 (2–16)	26 (6–52)	0.96 (0.95–0.97) <sup>c</sup>	0.98 (0.96–1.00)
Having pulmonary TB or pulmonary and extrapulmonary TB	10,015 (68)	10,112 (68)	1,243 (66)	8,772 (68)	0.91 (0.82–1.01)	0.92 (0.79–1.07)

BCG: Bacillus Calmette–Guérin; CI: confidence interval; DST: drug susceptibility testing; IQR: interquartile range; OR, odds ratio; SD: standard deviation; TB: tuberculosis.

<sup>a</sup> Data are presented as number (percentage), unless indicated otherwise.

<sup>b</sup> OR adjusted for sex, age, living in an urban area (Amsterdam, Rotterdam, The Hague, Utrecht), being born in the Netherlands/foreign born, being vaccinated with BCG, belonging to a risk group (i.e. a group with high risk of exposure to TB, such as drug users, asylum seekers, illegal residents), had previous treatment and had pulmonary TB.

<sup>c</sup>  $P < 0.05$ .

<sup>d</sup> Calculated for foreign-born patients.

<sup>e</sup> Data were missing for BCG vaccination (888 resistant isolates; 5,648 susceptible isolates) and previous TB treatment (268 resistant isolates; 1,464 susceptible isolates).

<sup>f</sup> Calculated for previously treated patients.

PDR cases were classified into three groups according to a classification model previously described [16]: (i) PDR cases with an *M. tuberculosis* strain that had a unique DNA fingerprint, as well as clustered cases without a potential source case (i.e. without a preceding pulmonary TB case in the cluster) were considered infected abroad or before 1993; (ii) clustered PDR cases with a potential source case, but without a confirmed or likely epidemiological link to a previous case in the cluster were considered possibly infected in the Netherlands; and (iii) clustered PDR cases with a potential source case and a confirmed or likely epidemiological link with a previous case in the cluster were considered definitely infected in the Netherlands. An epidemiological link was considered ‘confirmed’ when a social contact with another patient in the cluster had been documented in an interview with the respective patients or ‘likely’ if the patients visited the same place at the same time (without being aware of each other’s presence) [17].

### Statistical analysis

We used logistic regression analyses to assess the statistical significance of trends in resistance, with antituberculosis drug resistance as dichotomous outcome

variable and year of diagnosis as continuous independent variable. A trend was considered significant if the regression coefficient for year of diagnosis differed significantly from 0. A positive coefficient indicated an increasing trend and a negative coefficient indicated a decreasing trend. We calculated 95% confidence intervals (CIs) for drug resistance percentages over time, assuming the number of resistant samples was normally distributed.

Univariate and multivariate logistic regression analyses were used to examine which determinants were associated with resistance (dependent variables). Determinants evaluated were demographic variables – i.e. sex, age, living in an urban area (in one of the four largest cities in the Netherlands (Amsterdam, Rotterdam, The Hague, Utrecht), being foreign-born, length of time of residence in the Netherlands and belonging to a risk group (i.e. a group with a high risk of exposure to TB, such as drug users, asylum seekers, illegal residents), Bacillus Calmette–Guérin (BCG) vaccination, previous treatment history and having pulmonary TB.

Multivariate logistic regression was used to adjust for possible confounders. Variables with  $p < 0.05$  in the univariate analysis, as well as variables that were expected to be related to the outcome measure were used. In addition, differences between ADR and PDR cases were analysed using multivariate logistic regression with type of drug resistance (ADR/PDR) as dichotomous dependent variable and drug-specific resistance, age and being foreign-born as covariates. Crude and adjusted odds ratios (ORs) are presented with 95% CIs. All statistical analyses were performed using SPSS version 19. A significance level of 0.05 was used throughout.

## Results

During 1993 to 2011, 18,294 isolates were collected from 18,274 notified TB cases. A total of 15,601 isolates (85%) could be matched with the Netherlands Tuberculosis Register data, of which 14,959 (96%) were *M. tuberculosis* cultures. A total of 14,820 of these isolates (99%) had DST results and 1,890 (13%) of these strains showed resistance to at least one antituberculosis drug. Resistance was found in 1,500 (12%) of 12,678 new cases and 122 (23%) of all 539 previously treated patients. Resistance to isoniazid and streptomycin was most common, while rifampicin, ethambutol and pyrazinamide resistance and MDR-TB (defined as resistance to at least isoniazid and rifampicin) were less frequently observed (data not shown). The mean age of *M. tuberculosis*-positive TB cases was 41 years (SD: 20). Of the 14,959 *M. tuberculosis* isolates, 8,941 (60%) were from cases who were male; 5,218 (35%) lived in an urban area; 10,257 (69%) were foreign-born and 10,112 (68%) had pulmonary TB (including those with pulmonary TB and extrapulmonary TB). Of 8,376 patients with data on vaccination and resistance status, 4,182 (50%) were BCG vaccinated (Table 1).

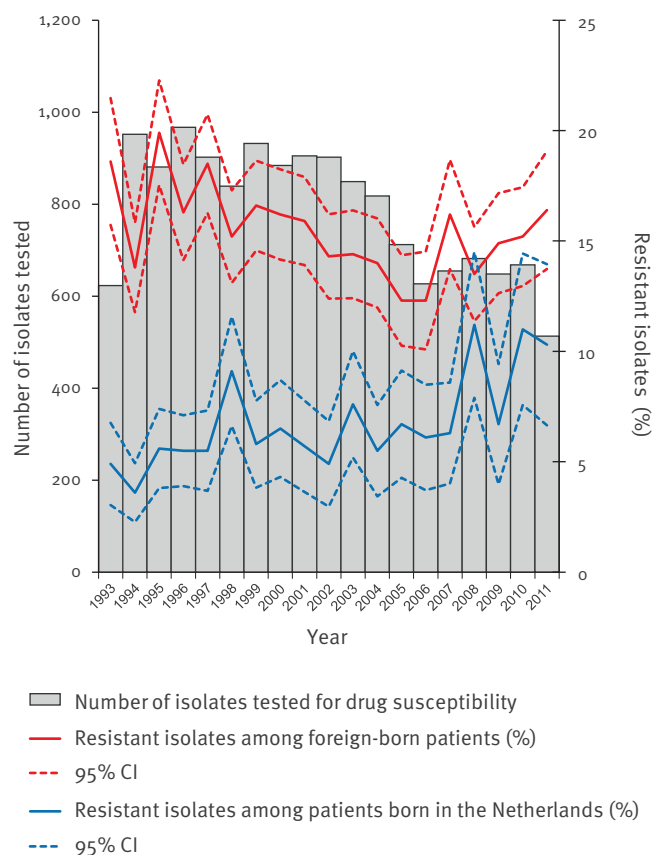
## Trends in drug resistance

The resistance rate was considerably higher in foreign-born TB patients than in patients born in the Netherlands (16% vs 6%,  $p < 0.001$ ). Among those who were foreign-born, trend analysis showed a slightly decreasing trend in the proportion of resistant isolates during 1993 to 2005 ( $p$  value for trend ( $p_{\text{trend}}$ ) = 0.01), followed by a significantly increasing trend until 2011 ( $p_{\text{trend}}$  = 0.01, Figure 1). However, the 95% CIs for 2005 and 2011 overlap slightly (10.3–14.3 and 13.7–19.1, respectively). Among patients born in the Netherlands, resistance increased from 5% (95% CI: 3.0–6.8) in 1993 to 10% (95% CI: 6.8–13.9) in 2011 ( $p_{\text{trend}}$  < 0.001) (Figure 1). For the total population, the proportion of isolates resistant to any TB drug fluctuated around 12% until 2005, but then increased significantly from 10.7% (95% CI: 9.1–12.3) in 2005 to 15% (95% CI: 12.6–17.0) in 2011 ( $p_{\text{trend}}$  < 0.001) (data not shown).

We found a significantly increasing trend for drug resistance, when excluding streptomycin resistance, from 7.1% (95% CI: 5.7–8.5) in 1993 to 11.1% (95% CI: 9.2–13.0) in 2011 (Figure 2,  $p_{\text{trend}}$  < 0.001). Streptomycin

**FIGURE 1**

Trend in the proportion of *Mycobacterium tuberculosis* isolates with resistance to at least one antituberculosis drug in the Netherlands, for patients born in the Netherlands and foreign-born patients, 1993–2011



CI: confidence interval.

Data from the National Reference Laboratory at the National Institute for Public Health and the Environment (RIVM). The number of isolates tested for drug susceptibility was lower in 2011 because local laboratories started to test for drug susceptibility as well. Not all isolates were sent to the National Reference Laboratory any more.

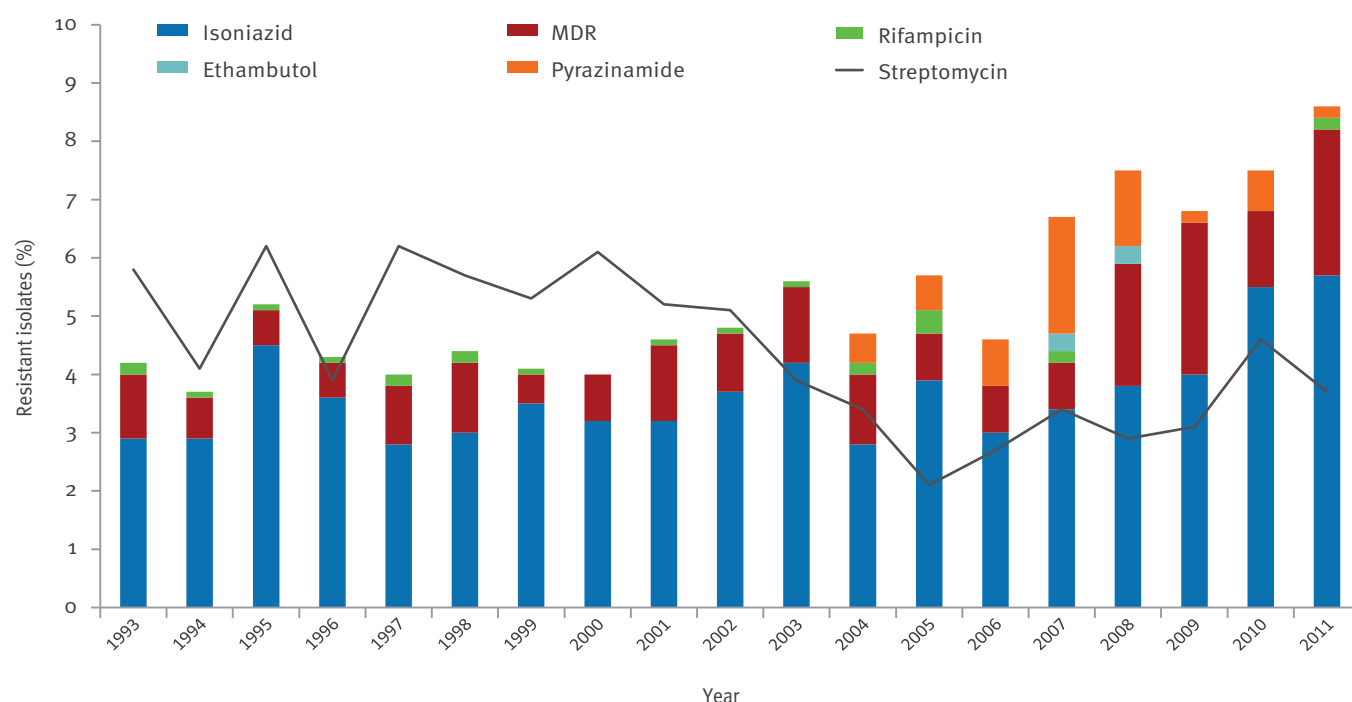
has not been used in the Netherlands since 1996, when new TB treatment guidelines were issued, and resistance to it decreased from 5.8% (95% CI: 4.5–7.1) in 1993 to 3.7% in 2011 (95% CI: 2.5–4.9) ( $p_{\text{trend}}$  < 0.001). In particular, the percentage of isolates with isoniazid resistance increased significantly from 2.9% (95% CI: 2.0–3.8) in 1993 to 5.7% in 2011 (95% CI: 4.3–7.1) (Figure 2,  $p_{\text{trend}}$  = 0.01). The same applied to MDR-TB: from 1.1% (95% CI: 0.5–1.7) in 1993 to 2.5% (95% CI: 1.5–3.5) in 2011 (Figure 2,  $p_{\text{trend}}$  < 0.001).

When analysing cases by country of birth, and excluding streptomycin resistance, resistance increased from 2.3% (95% CI: 1.0–3.6) in 1993 to 8.1% (95% CI: 4.8–15.4) in 2011 among cases born in the Netherlands and increased from 10.6% (95% CI: 8.3–12.9) in 1993 to 12.2% (95% CI: 9.8–21.8) in 2011 among foreign-born cases. Isoniazid resistance increased from 0.4% (95% CI: 0.0–0.9) in 1993 to 4.4% (95% CI: 1.9–11.1) in 2011 among cases born in the Netherlands and increased



**FIGURE 2**

Trends in the proportion of *Mycobacterium tuberculosis* isolates resistant to first-line antituberculosis drugs, the Netherlands, 1993–2011



MDR: multidrug resistance, defined as resistance to at least isoniazid and rifampicin.

Percentages calculated as the number of resistant isolates divided by the total number of isolates tested for drug susceptibility. Resistance to isoniazid and streptomycin is not shown (only mono-resistance and MDR).

from 4.7% (95% CI: 3.1–6.3) in 1993 to 6.1% (95% CI: 4.4–16.0) in 2011 among those who were foreign-born.

### Origin of drug resistance

Multivariate analyses showed that younger age, being foreign-born and previous TB treatment were independently related to resistance, while sex, living in an urban area, BCG vaccination, belonging to a risk group and having pulmonary TB were unrelated (Table 1). The significant univariate association between BCG vaccination and resistance may be explained by the fact that patients who were foreign-born were more likely to be vaccinated than those born in the Netherlands. Drug-resistant isolates from foreign-born cases more often expressed rifampicin resistance and MDR than drug-resistant isolates from cases born in the Netherlands (Table 2).

For 1,622 (86%) of all 1,890 patients with drug-resistant isolates, information on previous TB treatment was available. Of these, 122 (8%) had been treated previously and 1,500 (92%) had not been treated before. Consequently, 8% of all resistant cases for whom information on previous treatment was available were classified as ADR and 92% as PDR. This corresponds to 0.8% and 10% of all 14,959 TB cases analysed, respectively. In a multivariate analysis, we found that rifampicin resistance and MDR-TB were more associated with ADR

than PDR. We also found that ADR patients were older than PDR patients (Table 3).

The percentage of ADR cases was not different between patients born in the Netherlands and those who were foreign-born (Table 2). Time since previous treatment was much longer in ADR cases born in the Netherlands (mean: 22 years; SD: 19) than in foreign-born patients (mean 7 years; SD: 9);  $p < 0.001$ ). Two of 16 ADR patients born in the Netherlands were second-generation migrants as at least one parent had been born abroad. For nine ADR patients born in the Netherlands, the parents' country of birth was not registered. Of 92 foreign-born ADR patients with known date of entry into the Netherlands, 49 had previously been treated before entry and were thus considered to have acquired resistance abroad. A total of 29 foreign-born ADR patients had previously been treated after entry into the Netherlands and most probably acquired resistance or additional resistance in the Netherlands. For 14 foreign-born ADR patients, it was unknown where they acquired resistance, as the year of previous treatment coincided with the year of entry.

### Transmission of drug-resistant TB

Of all 14,959 isolates, 14,913 (99.7%) DNA fingerprints were generated. Due to the 15% mismatch with Netherlands Tuberculosis Register data, 454 clusters



**TABLE 2**

Comparison of *Mycobacterium tuberculosis* isolates with resistance to at least one antituberculosis drug from tuberculosis patients born in and outside the Netherlands, 1993–2011

Characteristic	Foreign-born	Born in the Netherlands	Crude odds ratio (95% CI)
	n=1,590	n=300	
Drug resistance	n (%) <sup>a</sup>	n (%) <sup>a</sup>	
Isoniazid	955 (60)	171 (57)	1.14 (0.89–1.46)
Rifampicin	183 (12)	16 (5)	2.31 (1.37–3.92) <sup>b</sup>
Streptomycin	1,025 (65)	176 (59)	1.28 (1.00–1.65)
Ethambutol	66 (4)	6 (2)	2.36 (1.00–5.58)
Pyrazinamide	61 (4)	19 (6)	0.64 (0.36–1.12)
Multidrug resistance (at least isoniazid and rifampicin)	156 (10)	11 (4)	2.86 (1.53–5.34) <sup>b</sup>
Other			
Previously treated patients with acquired drug resistance <sup>c</sup>	106 (8)	16 (6)	1.38 (0.80–2.37)
Mean number of years between diagnosis and previous treatment (SD) <sup>d</sup>	7 (9)	22 (19)	0.93 (0.91–0.96) <sup>b</sup>

CI: confidence interval; SD: standard deviation.

<sup>a</sup> Data are presented as number (percentage), unless indicated otherwise.

<sup>b</sup>  $P < 0.05$ .

<sup>c</sup> Data were missing for some previously treated patients (242 foreign-born; 26 born in the Netherlands).

<sup>d</sup> Calculated for patients with acquired drug resistance.

consisted of only one case and were excluded from further analysis. Of the resulting 14,459 isolates, 8,330 (58%) were unique, of which 1,675 (20%) were the first case in a cluster; and 6,129/14,459 (42%) were clustered cases.

The total number of clusters was 1,676 and the median cluster size was 6 (interquartile range: 3–19). Of the 1,676 clusters, 420 (25%) contained at least one case with resistant TB. Epidemiological cluster investigation was performed for 5,594 (91%) of all 6,129 clustered cases: an epidemiological link was confirmed in 1,674 (30%) clustered cases, likely in 923 (17%) clustered cases and could not be determined in 2,997 (54%) clustered cases.

PDR cases (n=1,445) were classified according to a transmission classification model (Figure 3): 129 cases (9%) were definitely infected in the Netherlands, 404 cases (28%) possibly and 912 cases (63%) were infected abroad or before 1993, when DNA fingerprinting was not systematically performed. PDR patients born in the Netherlands more often had clustered isolates than foreign-born PDR patients (132 (54%) vs 469 (39%),  $p < 0.001$ ) and were more likely to have a confirmed epidemiological link with a previous case in the cluster (54 (22%) vs 75 (6%),  $p < 0.001$ ) (data not shown).

## Discussion

This study, based on a large number of cases and molecular typing data from the Netherlands, covering many years, revealed that antituberculosis drug resistance has increased since 1993 in patients born in the

Netherlands and since 2005 in those foreign-born, and that resistance was more frequent among foreign-born patients. The increasing trend was mainly related to an increase in resistance to isoniazid, the cornerstone of first-line treatment. Furthermore, more than 90% of the drug resistance seen was a result of transmission. Our classification model suggests that transmission of resistant strains occurred in more than 60% of the cases before 1993 or abroad, and in 9% of the cases definitely in the Netherlands. Although ADR was rare, and mainly related to previous treatment abroad, in 45/122 cases it was associated with previous treatment failure in the Netherlands. Patient files should be retrieved and treatment history examined to gain more insight into the possible acquisition of resistance in the Netherlands.

The impact of the unexpected increase in antituberculosis drug resistance among patients born in the Netherlands is likely to be limited, because the majority of TB drug resistance is still mainly found in foreign-born patients, as reported previously [7].

The largest increase has been seen since 2005–06, when resistance among foreign-born patients has also been increasing. We suspect that the increase might be a result of enhanced migration from TB-endemic countries with high rates of TB drug resistance [18]. Concomitant intermingling of people born in the Netherlands and people with different ethnic backgrounds born outside the Netherlands might have resulted in the spread of drug-resistant TB. This might explain the increase in isoniazid resistance, as such

TABLE 3

Comparison of rates of resistance to various antituberculosis drugs in patients with acquired and primary drug resistance<sup>a</sup> among 1,622 patients diagnosed with drug-resistant tuberculosis, the Netherlands, 1993–2011

Characteristic	Acquired DR n=122	Primary DR n=1,500	Crude OR (95% CI)	Adjusted OR <sup>c</sup> (95% CI)
	n (%) <sup>b</sup>	n (%) <sup>b</sup>		
Drug resistance				
Isoniazid	86 (70)	882 (59)	1.67 (1.12–2.50) <sup>d</sup>	1.87 (0.83–4.18)
Rifampicin	36 (30)	130 (9)	4.41 (2.87–6.76) <sup>d</sup>	3.02 (1.43–6.38) <sup>d</sup>
Streptomycin	72 (59)	953 (64)	0.82 (0.57–1.20)	1.27 (0.67–2.43)
Ethambutol	17 (14)	47 (3)	3.86 (2.07–7.22) <sup>d</sup>	1.40 (0.57–3.47)
Pyrazinamide	8 (7)	53 (4)	1.49 (0.67–3.33)	1.32 (0.54–3.22)
Multidrug resistance (isoniazid and rifampicin)	32 (26)	109 (7)	4.53 (2.90–7.09) <sup>d</sup>	5.43 (3.39–8.65) <sup>d,e</sup>
Other				
Mean age in years (SD)	39 (16)	33 (15)	1.02 (1.01–1.03) <sup>d</sup>	1.03 (1.01–1.05) <sup>d</sup>
Foreign born	106 (87)	1,242 (83)	1.38 (0.80–2.37)	1.68 (0.66–4.30)
Median duration of stay in the Netherlands in years (IQR) <sup>f</sup>	2 (0–11)	4 (1–10)	1.00 (0.98–1.02)	0.99 (0.96–1.03)

CI: confidence interval; DR: drug resistance; IQR: interquartile range; OR: odds ratio; SD: standard deviation; TB: tuberculosis.

<sup>a</sup> Acquired drug resistance refers to resistance in previously treated TB cases; primary drug resistance refers to drug resistance in new TB cases.

<sup>b</sup> Data are presented as number (percentage), unless indicated otherwise.

<sup>c</sup> Adjusted for drug-specific resistance, age and being foreign-born.

<sup>d</sup>  $P < 0.05$ .

<sup>e</sup> Adjusted for age and being foreign-born.

<sup>f</sup> Calculated for foreign-born patients.

an increase has also been observed in other countries [19]. Furthermore, next to certain ‘host-related factors’ [20], *M. tuberculosis* in general might have gained a higher ability to withstand treatment, resulting in more persistent infections and higher rates of transmission to other people. Possibly, particular resistance mutations might be less deleterious than others or certain compensatory mutations might make up for any loss of fitness caused by the resistance mutation [21,22]. Borrell et al. demonstrated that the most common isoniazid resistance-conferring mutation in clinical settings reduced isoniazid activation while maintaining virulence in mice [23].

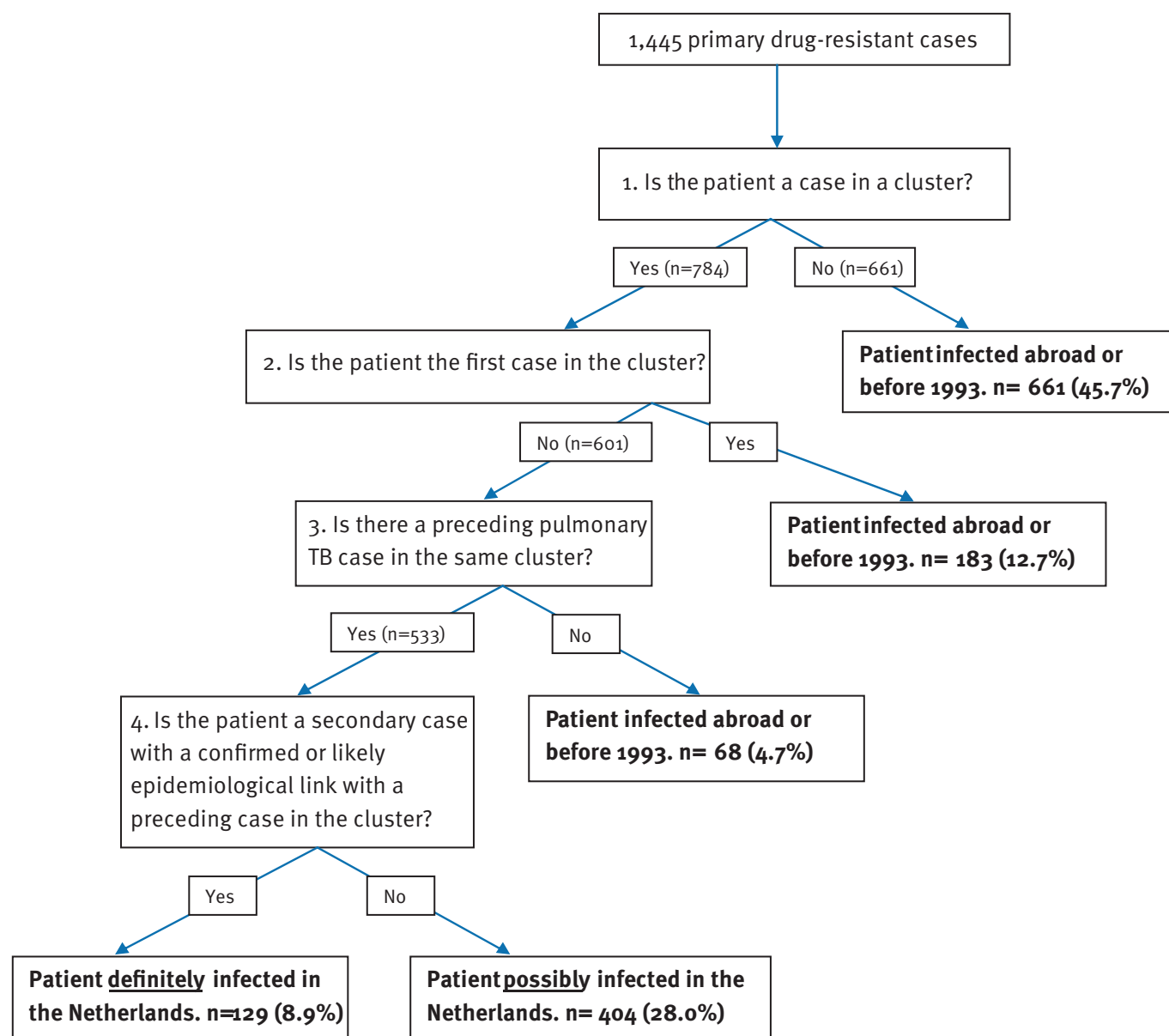
The pronounced increase in resistance to regularly used drugs is considered highly relevant as these are the cornerstone of treatment and may perhaps reflect a trend in other European countries where large numbers of migrants are received [3,24]. Although MDR-TB is diagnosed relatively rarely in the Netherlands [6], we should closely monitor the seemingly increasing MDR-TB trend, as its treatment is costly, complicated and enduring [3]. In Europe, trends in MDR-TB over the past five years have differed substantially by country [3]. The MDR-TB rate remained stable in European Union/European Economic Area (EU/EEA) countries (4.5% in 2011), while in non-EU/EEA countries, it increased from 20.3% in 2007 to 30.2% in 2011.

The 23% drug resistance among previously treated patients found in our study was similar to the median prevalence of resistance in previously treated patients found in a recent global surveillance project (25.1%) [8]. Our finding that resistance in *M. tuberculosis* was mainly the result of transmission was also in line with global findings [25]. In 129 of 784 clustered PDR cases, cluster investigation confirmed transmission of resistance in the Netherlands, despite the measures to prevent TB transmission such as contact tracing and screening of risk groups. Among foreign-born cases, clustering does not always reflect transmission in the Netherlands. In particular, clustered cases originating from the same foreign country might have been infected in their home country by strains of the predominant genotypes in those countries. This could also be an explanation for the low percentage of foreign-born PDR cases with a confirmed epidemiological link. In general, the proportion of patients definitely infected in the Netherlands is presumably underestimated, as transmission in a public space often remains unconfirmed in epidemiological cluster investigations. Furthermore, cluster investigation in foreign-born cases can be hindered by communication difficulties [26].

There are limitations associated with this study. Firstly, drug resistance trends could have been influenced by the change in DST method in 2004. However, the most

**FIGURE 3**

Classification model to determine the place of infection for tuberculosis patients with primary drug-resistant strains, the Netherlands, 1993–2011



pronounced increase in resistance was seen in the period since 2005, in which only the MGIT DST method was used. Moreover, DST at the National Reference Laboratory in the Netherlands has always been checked by WHO proficiency testing. Secondly, pyrazinamide susceptibility testing was less reliable before 2009 and may therefore have had an effect on the results. However, due to the rare occurrence of pyrazinamide resistance, this probably had little effect on the overall trends. Thirdly, misclassification of ADR and PDR cases could have occurred because the classification was based on self-reported treatment history. Fourthly, 14% of all patients with drug-resistant TB could not be classified as ADR or PDR at all, because their treatment history was unknown. Besides, ADR cases could have

been PDR cases if they were reinfected with a resistant strain that differed from the strain they were previously treated for. For instance, a previous study has shown that reinfection with a different strain occurred in 16% of all patients with Dutch nationality who had been infected before 1981 [27]. Additionally, ADR patients born in the Netherlands whose parents had been born abroad could have acquired resistance in their parents' country of origin, when visiting friends and family. The same may apply to patients who may have worked in a high TB burden country. On the other hand, the 14 foreign-born ADR cases with unknown place of previous treatment could have acquired resistance in the Netherlands. Lastly, for 454 patients who were part of clusters whose data could not be matched to that of

the National Reference Laboratory, the place of transmission could not be determined.

In conclusion, the increase in resistance of *M. tuberculosis* among patients born in the Netherlands and the recent increase among foreign-born patients have not led to an increase in the incidence of TB in the Netherlands, as the incidence has remained stable over the last few years [6]. With a high degree of transmission of resistant strains abroad and a large proportion of *M. tuberculosis* drug resistance among migrants, the problem of resistance in the Netherlands is closely related to the resistance problems in the migrants' countries of origin. This highlights the importance of early detection of TB, resistance screening and treatment programmes, especially in migrants originating from high-endemic countries with a high resistance rate. In these countries, preventive measures such as improved case detection, individualised treatment and improved drug supply and distribution could reduce the risk of acquiring resistance and its subsequent transmission. Our findings may be representative of the situation in other low-endemic European countries with a relatively large proportion of migrants. Generally, only little is known about trends in resistance as testing and reporting is currently not sufficiently frequent or complete in many countries. The capacity to respond adequately to the threat of drug-resistant TB requires more detailed information on the magnitude of this problem. Therefore more research is needed in other European countries. Resistance monitoring and surveillance remain highly important activities.

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## Conflict of interest

None declared.

## Authors' contributions

CR participated in the coordination of the study, performed the statistical analyses and had the lead in drafting the manuscript. AvG participated in the design of the study, in performing the statistical analyses and drafting the manuscript. GdV was involved in developing the transmission classification model and in drafting the manuscript. CE participated in the design of the study and drafting the manuscript. JvR participated in the coordination of the study. HKA participated in performing the statistical analyses and drafting the manuscript. HdN contributed to the drafting of the manuscript. MK

was involved in the design of the study and was responsible for the RFLP and VNTR typing of the isolates. DvS participated in the design and coordination of the study and was responsible for the RFLP and VNTR typing of the isolates and participated in drafting the manuscript. All authors read and approved the final manuscript.

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# Transmission of multidrug-resistant tuberculosis in a low-incidence setting, Switzerland, 2006 to 2012

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The goal of the present study was to examine the transmission dynamics of multidrug-resistant tuberculosis (MDR-TB) in Switzerland. Between 2006 and 2012, a total of 49 MDR-TB cases were reported to the Swiss Federal Office of Public Health, 46 of which were of foreign origin. All 49 initial strains were evaluated by molecular epidemiologic methods at the Swiss National Reference Centre for Mycobacteria. In 43 strains, unique DNA fingerprint patterns were identified. Twelve strains were grouped into six clusters. Data from contact tracing suggest likely in-country transmission in four clusters, mostly among close contacts. In the remaining two clusters, no contact tracing data were available, but the identified genotypes were known to be prevalent in the countries of origin of the patients, suggesting the possibility that the infection was acquired there. While most MDR-TB cases are imported to Switzerland, at least four of the 49 MDR-TB cases were due to transmission within the country. The imported cases, however, did not lead to secondary cases outside the circles of close contacts. The results also indicate that prevention of MDR-TB transmission among immigrants may require closer monitoring.

## Introduction

One of the most challenging concerns in tuberculosis control programmes, both in high and low-prevalence settings, is the emergence and transmission of multidrug-resistant tuberculosis (MDR-TB), by definition resistant at least to isoniazid and rifampicin [1]. The World Health Organization estimates 450,000 cases of MDR-TB to have occurred in 2012 worldwide, corresponding to 5.2% of all TB cases [2]. In Switzerland, the incidence of TB decreased steadily over many decades and seems to have reached a plateau in recent years at 7.0 per 100,000 population [3]. In the period 2005 to 2011, 71% of all TB cases in Switzerland were foreign-born. The proportion of culture-positive cases was 82.2% (3,093 of 3,763 cases) and 3,069 of the 3,093 culture-positive cases had susceptibility testing results, of which 47 (1.5%) had MDR-TB [3].

The goal of the present study was to analyse the transmission dynamics of MDR-TB in Switzerland by examining all MDR-TB strains identified in the country over a seven-year period using molecular methods and contact investigations in clustered cases.

## Methods

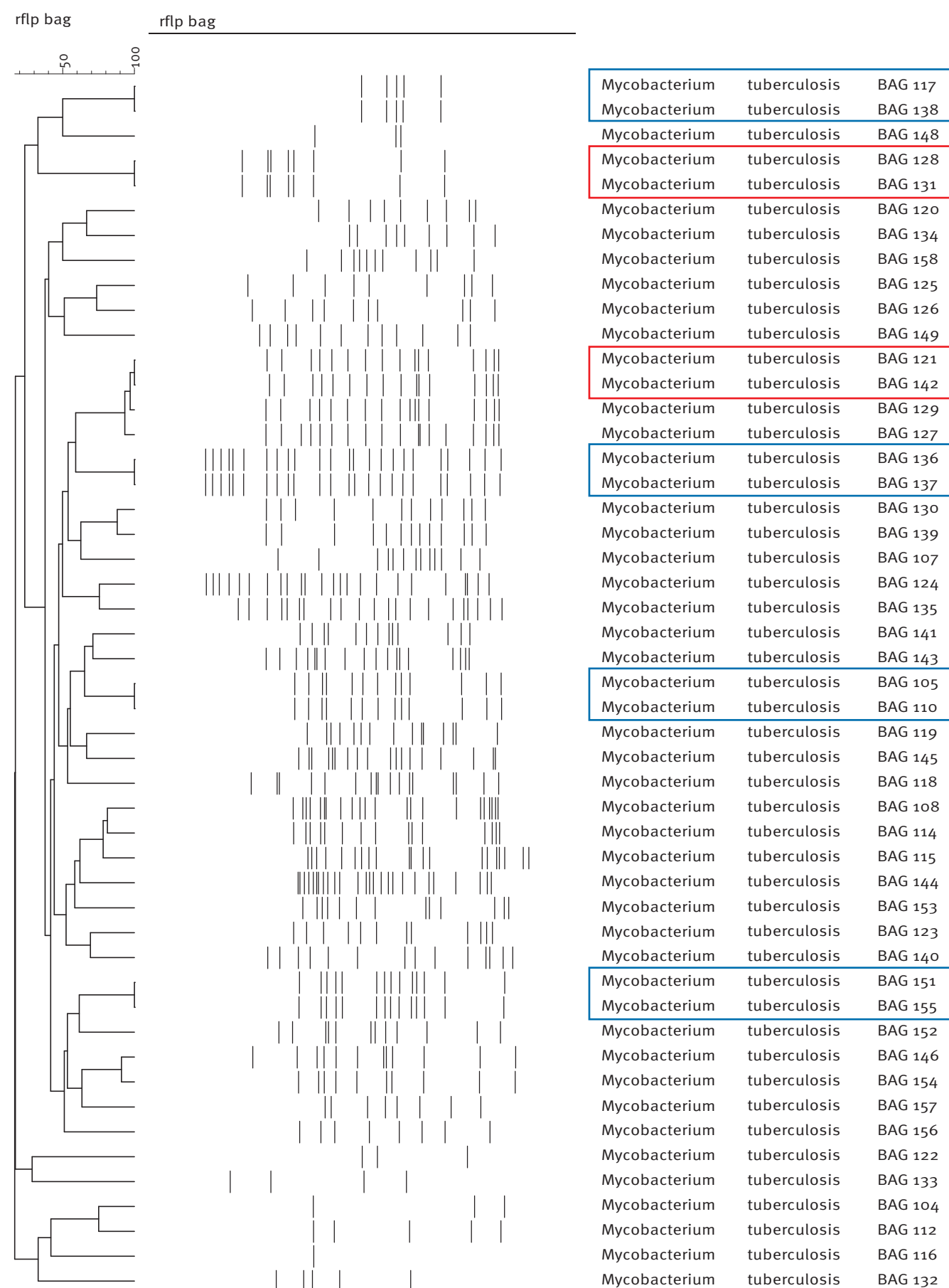
Since 1997, all laboratories in Switzerland have been submitting MDR-TB strains isolated in the country to the National Reference Centre for Mycobacteria (NZM). Cases were defined as MDR-TB when the isolate was resistant to at least isoniazid at 1.0 mg/mL and rifampicin at 1.0 mg/mL in the MGIT 960 system (Becton, Dickinson and Company) [4]. In the present study, we examined all initial MDR-TB isolates submitted to the NZM between January 2006 and December 2012. All strains (one strain per patient) were characterised by extensive conventional and molecular drug susceptibility testing as described earlier [4]. All isolates were also evaluated by IS6110 DNA restriction fragment length polymorphism (RFLP) fingerprinting, spoligotyping and 24-locus mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) as described previously [5-8]. Clusters were defined as groups of at least two patients with *Mycobacterium tuberculosis* strains showing identical IS6110 RFLP (same number of IS6110 bands at identical positions, position tolerance 1.2%), spoligotype and MIRU-VNTR patterns [7,8]. Baseline epidemiological data were obtained from the Swiss Federal Office of Public Health. Contact tracing was conducted by the local health authorities of the respective cantons. Results of the contact investigations were made available by the Lung Associations of Zurich and Bern ('Lunge Zürich' and 'Lungenliga Bern') acting on behalf of the respective health authorities.

## Results

Between 2006 and 2012, a total of 49 cases of MDR-TB were reported to the national surveillance system. None of the strains were extensively drug-resistant (XDR) isolates. Initial isolates of all these patients were submitted to the reference laboratory. Forty-six

## FIGURE

IS6110 fingerprint patterns of the 49 patients identified with multidrug-resistant tuberculosis and the 12 individuals in six clusters with identical fingerprints, Switzerland, 2006 and 2012 (n=49)



Blue: Clusters of patients with direct contact; red: clusters of patients for whom transmission in Switzerland is not probable.

patients were foreign-born and/or of foreign nationality. Of the three Swiss nationals, one had been living in Thailand and one was the teenage child of an MDR-TB patient born in sub-Saharan Africa, while no significant information could be identified on the exposure of the third individual.

Altogether, we identified 43 different DNA fingerprint patterns. Twelve of the 49 strains were grouped in six clusters with identical fingerprints, while 37 patients had individual fingerprints (Figure). Contact investigations of clustered patients confirmed epidemiological links in four clusters (Figure, highlighted in blue). First- and second-line drug susceptibility patterns were identical in all four clusters. All source cases of the four clusters were sputum smear-positive. In the remaining two clusters, the closest epidemiological link that could be confirmed was the geographical origin.

In the first cluster, the transmission event was identified among Tibetan immigrants and was from an adult to a child of pre-school age born in Switzerland. The source case was a friend of the family who regularly met and supervised the child. The primary contact investigation of the index case had not pointed to the child, who was diagnosed 10 months later and died of TB meningitis while under standard first-line treatment awaiting drug susceptibility testing results.

In the second cluster, transmission also occurred in Tibetan immigrants who were living together in Switzerland and had not met before their arrival to the country. Four weeks after the identification of the index case, asymptomatic active disease was detected radiologically in the contact during the contact investigation.

The third cluster was most likely the result of transmission between two immigrants from Turkey and Kosovo attending the same language school for several hours a day over many weeks. In this cluster, the contact tested tuberculin-negative eight weeks after the last exposure, but became symptomatic with pulmonary TB three years later.

The fourth cluster was due to transmission from a parent to their teenage child. The index patient had immigrated from Sub-Saharan Africa to Switzerland 14 years earlier. Pulmonary TB was identified in the asymptomatic child during the contact investigation.

The fifth molecular cluster consisted of two Ethiopians. One case was diagnosed with spinal TB one year before the second case was diagnosed with pulmonary TB and unknown sputum smear status. The contact investigation of the pulmonary case did not establish an epidemiological link and the investigation could not be re-opened for further investigations by the time the molecular epidemiological results became available.

The sixth cluster consisted of two patients from Ukraine and Estonia, diagnosed in 2007 and 2011: a tourist and

an asylum seeker, respectively, with different resistance profiles. Both patients could not be located any more for initiation of treatment. No additional data of contact tracing are available.

Molecular epidemiological testing of the fifth and sixth cluster (Figure, highlighted in red) showed the presence of strains with genotypes that were highly prevalent in the home countries of these patients: the ill-defined T family in Cluster 5, and the Beijing genotype in Cluster 6 [8,9].

Thus, at least four secondary cases (Clusters 1 to 4, highlighted in blue in Figure) were due to transmission within Switzerland during the examined period, corresponding to 8% of all MDR-TB cases in the country.

## Discussion

This report is providing molecular epidemiological insight into the transmission dynamics of MDR-TB in a low-incidence setting over a seven-year period. We have identified clustering in a quarter of the 49 MDR-TB strains that represent all MDR-TB cases reported nationwide in the period from 2006 to 2012. Transmission leading to secondary cases was confirmed by conventional contact tracing in four of the 49 cases. Transmission occurred mainly among persons living together or otherwise spending significant time together in a closed room on a regular basis over several weeks.

Comparable studies have been carried out in other resource-rich, low-incidence settings with similar population sizes and with a majority of TB cases occurring in immigrants (Table). A similar proportion of cases with recent transmission (7%; 2 of 29 MDR-TB cases) was found in Denmark over the period from 1992 to 2007 [10]. In a long-term and prospective follow-up of contacts exposed to MDR-TB patients in Victoria, Australia, the transmission rate was 5% (2 of 40 cases) in the period from 2002 to 2010 [11]. However, in a study in Galicia in the period from 1998 to 2004, with the vast majority of MDR-TB patients of Spanish origin, 53% of MDR-TB patients (30 of 57) were grouped in four clusters [12]. Unfortunately, drug susceptibility testing was not performed routinely on all clinical isolates in the latter study so that only about half of the MDR-TB cases estimated to have occurred were identified, thus possibly overestimating the proportion of clustering. Approximately half of the clustered cases could be attributed to recent transmission (two probable outbreaks, one of them nosocomial among patients and healthcare workers) [11]. In a German study representing an estimated 75% of all MDR strains occurring country-wide in 1995 to 2001, the rate of clustered MDR-TB cases was reported to be 49.4% (214 of 433 patients) [13]. Epidemiological links were established among 18.2% of the clustered patients (39 of 214), which corresponds to a proportion of cases with recent transmission of 5.8% (25 of 433). Taken together, these findings demonstrate that confirmed transmission of

**TABLE**

Proportion of multidrug-resistant tuberculosis cases with transmission to subsequent cases in different low-incidence settings

Reference	Geographical setting	Time period	Number of cases	Proportion of clustered cases	Proportion of confirmed transmissions with subsequent cases
[9]	Denmark	1992–2007	29	17%	7%
[10] <sup>a</sup>	Victoria, Australia	2002–2010	40	33%	5%
[11]	Galicia, Spain	1998–2004	57	53%	28%
[12]	Germany	1995–2001	433	49%	6%
This paper	Switzerland	2006–2012	49	25%	8%

<sup>a</sup> This study [10] calculated the proportion of confirmed transmission based on prospective and long-term follow-up of contacts, while the other studies calculated the proportion of multidrug-resistant tuberculosis cases attributable to recent transmission.

MDR-TB with subsequent progression to disease is not infrequent in settings with low incidence and with cases predominantly in immigrants.

MDR-TB may or may not differ from drug-susceptible TB in terms of transmissibility. At present, the effects of drug resistance on transmission of tuberculosis are only partly understood. On the one hand, delays in initiating adequate therapy may prolong infectiousness of a patient. On the other hand, drug resistance-associated mutations may lead to reduced fitness of the bacterium, which may decrease the chance of transmission [14–16]. As in other studies in small geographical areas with large proportions of patients from elsewhere, some of the contacts in our study may have remained in the area only for a limited period of time. This reduces the chances of the contact still being in the area, or in the country, when signs of active disease develop. Our study may thus underestimate the true extent of onward transmission particularly in population groups in which social contacts tend to be limited to fellow migrants. However, our results that all confirmed MDR-TB transmissions were identified in immigrants are in line with findings of a recent systematic review which found that transmission of TB from foreign-born patients does not have a significant influence on the TB situation in native-born populations in the European Union or European Economic Area [17]. A further limitation is that seven years of observation is a short time, seeing as cluster studies tend to underestimate clustering at the beginning and at the end of the study period, when source cases or secondary cases, respectively, are not included. The strength of our study is the completeness of the dataset, as all microbiologically confirmed MDR-TB cases of the country were assessed.

In conclusion, our findings show that considerable transmission of MDR-TB, leading to secondary cases, occurred in Switzerland between 2006 and 2012. However, our estimated transmission rate of 8% may be an underestimate of the true situation because the majority of MDR-TB cases were detected among

immigrants and their foreign contacts, who often leave the country before active disease develops. Our study also indicates that prevention of MDR-TB transmission among immigrants may warrant closer monitoring. Since treatment and management of each MDR-TB case may be complicated with uncertain outcomes, appropriate measures and structures must be in place so that cases can be handled adequately and timely, and transmission can be prevented.

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### Conflict of interest

None declared.

### Authors' contributions

Akos Somoskovi: study design, data and literature analysis, writing of manuscript;

Peter Helbling: data and literature analysis, writing of manuscript; Vanessa Deggim: data and literature analysis; Rico Hömke: molecular and conventional mycobacteriology testing, data analysis; Claudia Ritter: molecular and conventional mycobacteriology testing, data analysis; Erik C. Böttger: study design, data and literature analysis, writing of manuscript.

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# Geographical heterogeneity of multidrug-resistant tuberculosis in Georgia, January 2009 to June 2011

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In 2011, Georgia, in the Caucasus, reported that 11% of new and 32% of previously treated tuberculosis (TB) cases nationally had multidrug-resistant TB (MDR-TB). To help understand the mechanisms driving these high risks of drug-resistance and plan for targeted interventions, we identified geographical variability in the MDR-TB burden in Georgia and patient-level MDR-TB risk factors. We used routinely collected surveillance data on notified TB cases to estimate the MDR-TB incidence/100,000 people and the percentage of TB cases with MDR-TB for each of 65 districts and regression modelling to identify patient-level MDR-TB risk factors. 1,795 MDR-TB cases were reported (January 2009–June 2011); the nationwide notified MDR-TB incidence was 16.2/100,000 but far higher (837/100,000) in the penitentiary system. We found substantial geographical heterogeneity between districts in the average annual MDR-TB incidence/100,000 (range: 0.0–5.0 among new and 0.0–18.9 among previously treated TB cases) and the percentage of TB cases with MDR-TB (range: 0.0%–33.3% among new and 0.0%–75.0% among previously treated TB cases). Among treatment-naïve individuals, those in cities had greater MDR-TB risk than those in rural areas (increased odds: 43%; 95% confidence interval: 20%–72%). These results suggest that interventions for interrupting MDR-TB transmission are urgently needed in prisons and urban areas.

## Introduction

In 2011, there were an estimated 8.7 million newly infected cases of tuberculosis (TB) worldwide and 1.4 million deaths attributed to TB [1]. The appearance and spread of forms of *Mycobacterium tuberculosis* that are resistant to drugs in the standardised TB treatment regimen are threats to effective TB control. People suffering from multidrug-resistant TB (MDR-TB, i.e. TB that is resistant to at least isoniazid and rifampicin) require longer treatment with second-line drugs (SLD) that are more expensive and more toxic than those in the standard TB drug regimen; even in settings where

individuals receive the best available care, poor outcomes are common [2].

Global estimates indicate that 3.7% of new TB cases and 20% of previously treated TB cases have MDR-TB [1], but these averages mask substantial geographical heterogeneity in risk. Countries of the eastern part of the World Health Organization (WHO) European Region have reported percentages of TB cases with MDR-TB several times higher than countries elsewhere in the world [1,3]. Georgia is a country of approximately 4.5 million people [4] located in the Caucasus and, like many countries in this region, it is experiencing a MDR-TB crisis. In response to a growing regional appreciation for the severity of this issue, Georgia made further investment in their commitment to provide universal access to diagnosis and treatment for drug-resistant TB [5] and begin routine surveillance to monitor drug resistance in 2006. As of 2011, Georgia was one of only six countries (among the 27 high burden MDR-TB countries) to have routine TB surveillance in place (i.e. nationwide, continuous, real-time notifications of all diagnosed drug resistant TB cases as opposed to sub-national reporting and/or periodic surveys) [1]. In 2011, 11% of notified new TB cases and 32% of notified previously treated TB cases in Georgia had MDR-TB and the national estimated TB incidence rate was 125/100,000 [1].

Statistics on MDR-TB burden are usually reported at the country level and few countries have sufficient detailed spatial resolution of data to examine local heterogeneity of MDR-TB burden [6]. Previous work has indicated that even in countries where TB patients have a very high overall risk of MDR-TB, the spatial variation in this risk can be dramatic, indicating potential opportunities for prioritising earliest responses and confirmatory studies to areas deemed at highest risk [6–8]. Here, we present spatial analyses of MDR-TB risk and incidence across Georgia in an attempt to identify

areas of relative high risk and/or incidence of MDR-TB among both new and previously treated TB cases. We also evaluate patient-level risk factors for MDR-TB amongst these patients.

## Methods

### Data sources

We analysed two TB surveillance databases that contained information on: (i) all TB cases notified in Georgia between January 2009 and December 2011 (database 1) and (ii) all patients that were hospitalised and initiated on SLD in Georgia between January 2009 and December 2011 (database 2).

In Georgia, all suspected TB cases based on clinical findings and chest radiography receive sputum smear microscopy at their local TB facility. All sputum samples, regardless of their microscopy findings, are then transported to the closer of two laboratories in the cities of Tbilisi or Kutaisi for culture testing. A cold chain is maintained and transportation time does not exceed 24 hours. Both laboratories have passed External Quality Assurance conducted by the Supranational Reference Laboratory in Antwerp, Belgium and are the only two laboratories in Georgia that perform TB culture testing. TB diagnosis is confirmed by positive microscopy and/or culture testing; both tests are required for all TB suspects under the national TB policy. Drug susceptibility testing (DST) is required under national policy for all culture positive sputum samples and is carried out only in the laboratory in Tbilisi. Culture and DST are done by conventional Lowenstein-Jensen solid media and/or broth-based culture methods using the MGIT 960 system (BD, Sparks, MD, USA) [9].

When a TB case is notified, a paper form is completed by the attending physician containing demographic data on the case (including information such as age, sex, previous treatment status and previous detention status). These forms are sent to the regional database manager and entered into the online TB notifications database (database 1). When culture and DST results are available, these are sent in paper forms to the database manager in Tbilisi and entered into the same online TB database.

All TB patients in Georgia are initially hospitalised and remain there until their smear microscopy results are negative and their clinical condition is stable. If patients have MDR-TB or polydrug-resistant TB confirmed by DST or do not receive culture and/or DST but are suspected MDR-TB cases such as household contacts of an MDR-TB case, they are hospitalised and initiated on SLD. Once these patients have started treatment, they are entered onto a second TB database (database 2). The average length of stay in hospital is two months for non-MDR-TB patients and three to four months for patients on SLD.

Georgia achieved universal access to TB diagnosis and treatment by the end of 2009. Since then, all notified TB (non-MDR-TB and MDR-TB) patients have had access to treatment as per WHO recommendations [10].

For our spatial analysis, each patient is indexed by the TB outpatient facility located within their district/city of residence (as defined in the National Census [4], a total of 72 districts and cities); these districts/cities are further aggregated into 12 administrative regions. There are seven facility codes within the city of Tbilisi, which we grouped together for our analysis. All patients diagnosed within the penitentiary system were attributed to the penitentiary system and were excluded from the spatial analysis. In addition, the region Abkhazia (consisting of 6 districts) and the district Java do not notify TB patients in either TB surveillance database. These seven districts were excluded and therefore the analysis focused on a total of 65 districts/cities. Five of the districts are classed as 'cities' and therefore our definition of 'urban areas' included these five cities. All other areas were classed as 'rural areas'.

### Statistical analysis

#### Estimation of the absolute number of multidrug-resistant tuberculosis notifications and multidrug-resistant tuberculosis incidence

We aimed to report the number of MDR-TB cases that were notified between 2009 and 2011 and confirmed through DST. In practice, not all culture and DST results were entered in the TB notifications database (database 1); 33.5% of cases recorded in database 1 did not have any information on culture results recorded in that database. Therefore, we used database 2 (hospitalisations for second-line treatment) as our primary source to calculate the number of MDR-TB notifications (93.4% of cases in database 2 had sufficient DST to diagnose or rule-out MDR-TB). From this database, we extracted the number of MDR-TB cases diagnosed and confirmed through DST. However, since it is a database of cases that initiated treatment between January 2009 and December 2011, some cases that were diagnosed in late 2011 and had not initiated treatment by the end of 2011 were not yet entered in this database. Hence, to minimise such errors, we included cases that were diagnosed between January 2009 and June 2011 inclusive. Additionally, since this database included all those cases that were hospitalised, it excluded some MDR-TB patients who were not hospitalised (for example, due to death or default prior to hospitalisation or due to lack of available treatment during 2009 before universal access became available) or were missing from database 2 due to data entry errors. Therefore, we also identified all DST-confirmed MDR-TB cases that were recorded between January 2009 and June 2011 in the TB notifications database (database 1) that were not found in the SLD hospitalisations database (database 2) and included these in our count of MDR-TB diagnoses. If one individual had two TB episodes during the study period, these would be considered as

TABLE 1

Burden of multidrug-resistant tuberculosis (MDR-TB) in Georgia, January 2009–June 2011

Indicator	Entire country (including penitentiary system, urban and rural areas) <sup>a</sup>			Penitentiary system			Urban areas			Rural areas		
	New	Previously treated	Total <sup>b</sup>	New	Previously treated	Total <sup>b</sup>	New	Previously treated	Total <sup>b</sup>	New	Previously treated	Total <sup>b</sup>
Number of notified MDR-TB cases	359	1,425	1,795	82	394	477	170	670	848	107	356	465
MDR-TB of all with DST <sup>c</sup> % (n/N); 95% CI	10.2 (538/5,261); 9.4–11.1	32.7 (521/1,595); 30.4–35.1	15.5 (1,075/6,931); 14.7–16.4	9.8 (107/1,094); 8.2–11.6	37.8 (178/471); 33.5–42.3	18.1 (287/1,582); 16.3–20.1	13.5 (264/1,950); 12.1–15.1	32.6 (172/528); 28.7–36.7	17.6 (441/2,502); 16.2–19.2	7.5 (165/2,193); 6.5–8.7	28.7 (169/589); 25.2–32.5	12.1 (342/2,815); 11.0–13.4
Average annual notified MDR-TB incidence per 100,000 people	3.2	12.8	16.2	143.9	691.5	837.1	4.2	16.4	20.7	1.5	5.1	6.6

CI: confidence interval; DST: drug susceptibility testing.

<sup>a</sup> Statistics for the entire country include five additional cases diagnosed in a psychiatric institution.<sup>b</sup> Totals may include cases for whom previously treated status was unavailable.<sup>c</sup> Exact binomial confidence interval using method of Agresti and Coull [18].

two incident cases. This was to correctly capture the burden placed on TB services since each incident case needs to be diagnosed and placed on treatment each time.

To estimate average annual MDR-TB incidence per 100,000 population, we divided our count of MDR-TB diagnoses by the population of Georgia in the same time period as reported in the National Census [4]. The most recent National Census was conducted in 2002. However, population numbers are recalculated annually using methods such as telephone interviews in order to fully account for variation in population changes at a local level and events that have resulted in substantial population movements. In addition to overall incidence, we estimated incidence rates by age and sex. We also estimated the average annual incidence of MDR-TB per 100,000 population for each district. All measures were estimated for new TB cases (i.e. those who have either previously received treatment for less than one month in total or never) and previously treated TB cases (i.e. those who have previously received one month or more TB treatment) separately [11]. Separate analyses of these two groups is important since resistance among new cases is the result of transmission of MDR-TB whereas resistance among previously treated cases may reflect either transmitted resistance or resistance acquired during previous exposure to TB drugs.

#### Estimation of the percentage of tuberculosis cases with multidrug-resistant tuberculosis

While measures of incidence provide information on the resource needs for programmes, measures of risk also provide useful additional information as they indicate the probability that individual patients diagnosed with disease will harbour an MDR-TB infection. To produce valid estimates of the percentage of TB cases that had MDR-TB, we needed to ensure that we estimated this risk from a population in whom DST was systematically employed without regard to actual risk of drug-resistance. Since, during the period of this analysis, DST was a routinely employed test, we used data extracted from the TB notifications database (database 1) under the assumption that there was no systematic bias in terms of risk of resistance between those TB cases that did or did not have data on DST results. We also estimated the percentage of TB cases that had MDR-TB for each district using database 1. All measures were estimated for new and previously treated TB cases separately.

#### Patient-level risk factors for multidrug-resistant tuberculosis

We developed logistic regression models (for new and previously treated cases separately) to identify risk factors associated with MDR-TB diagnosis. As we did when estimating the percentage of TB cases with MDR-TB, we used only the data from the TB notifications database (database 1) under the assumption that there was no systematic bias in the availability of culture and DST results in each group (i.e. cases (confirmed MDR-TB

**TABLE 2**

Age and sex-adjusted rates of notified multidrug-resistant tuberculosis (MDR-TB) incidence per 100,000 people, Georgia, January 2009–June 2011

Age group (years)	MDR-TB								
	Among new TB cases (n=359)			Among previously treated TB cases (n=1,425)			Among all TB cases (n=1,795)		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
0–4	0.9	1.3	1.1	1.2	0.0	0.6	2.0	1.3	1.7
5–14	0.8	0.8	0.8	0.8	0.5	0.6	1.7	1.4	1.5
15–24	6.2	2.1	4.2	18.5	6.9	12.8	24.9	9.1	17.1
25–34	11.4	2.5	6.9	43.9	9.9	26.8	55.5	12.5	33.9
35–44	7.6	0.6	4.0	44.2	5.0	23.8	52.0	5.6	27.8
45–54	5.5	1.3	3.2	27.9	2.9	14.5	33.9	4.2	18.0
55–64	3.0	1.0	1.9	20.0	2.0	10.1	23.0	2.9	12.0
≥64	2.5	0.7	1.4	4.7	2.0	3.0	7.3	2.8	4.5
<b>Total</b>	<b>5.4</b>	<b>1.3</b>	<b>3.2</b>	<b>22.6</b>	<b>4.1</b>	<b>12.9</b>	<b>28.1</b>	<b>5.5</b>	<b>16.2</b>

TB: tuberculosis.

cases) and controls (cases confirmed without MDR-TB)). A full model was constructed including all potential explanatory variables to obtain fully adjusted odds ratios. Potential explanatory variables that we examined included demographic data (e.g. age and sex), socio-economic data (e.g. occupation and previous detention status) and TB-related data (e.g. previous TB treatment outcome and disease location). A backwards elimination method was used to identify variables that were statistically significantly associated with MDR-TB diagnosis. In addition, any non-statistically significant variable which, on removal, altered other parameter estimates substantially (>10%) remained in the model to ensure full adjustment for confounding.

## Results

### Burden of multidrug-resistant tuberculosis in Georgia

Between January 2009 and June 2011, we identified 1,795 incident cases of MDR-TB confirmed through DST (Table 1) (1,370 from database 2 and an additional 425 were identified in database 1). Average annual MDR-TB notified incidence was 16.2 per 100,000 (3.2 new MDR-TB cases per 100,000 and 12.8 previously treated MDR-TB cases per 100,000). Incidence rates varied substantially by age and sex with the highest rates in men aged between 25 and 34 years (average of 55.5 annually per 100,000, Table 2). The estimated percentage of TB cases with MDR-TB was 15.5% (95% confidence interval (CI): 14.7%–16.4%); an estimated 10.2% of new and 32.7% of previously treated TB cases had MDR-TB (Table 1). While the percentages of TB cases diagnosed in the penitentiary system with MDR-TB were similar to those in the civilian population, the incidence of notified MDR-TB was considerably higher at 837 cases

per 100,000 (477 cases total, representing 27% of all MDR-TB notifications in Georgia) (Table 1).

### Geographical heterogeneity in multidrug-resistant tuberculosis risk and incidence

There was substantial variation in the MDR-TB burden by district. The incidence of notified MDR-TB per 100,000 varied from 0.0 to 5.0 for new cases and from 0.0 to 18.9 for previously treated cases (Figure 1). The percentage of TB cases with MDR-TB varied from 0.0% to 33.0% among new TB cases and from 0.0% to 75.0% among previously treated TB cases (Figure 2).

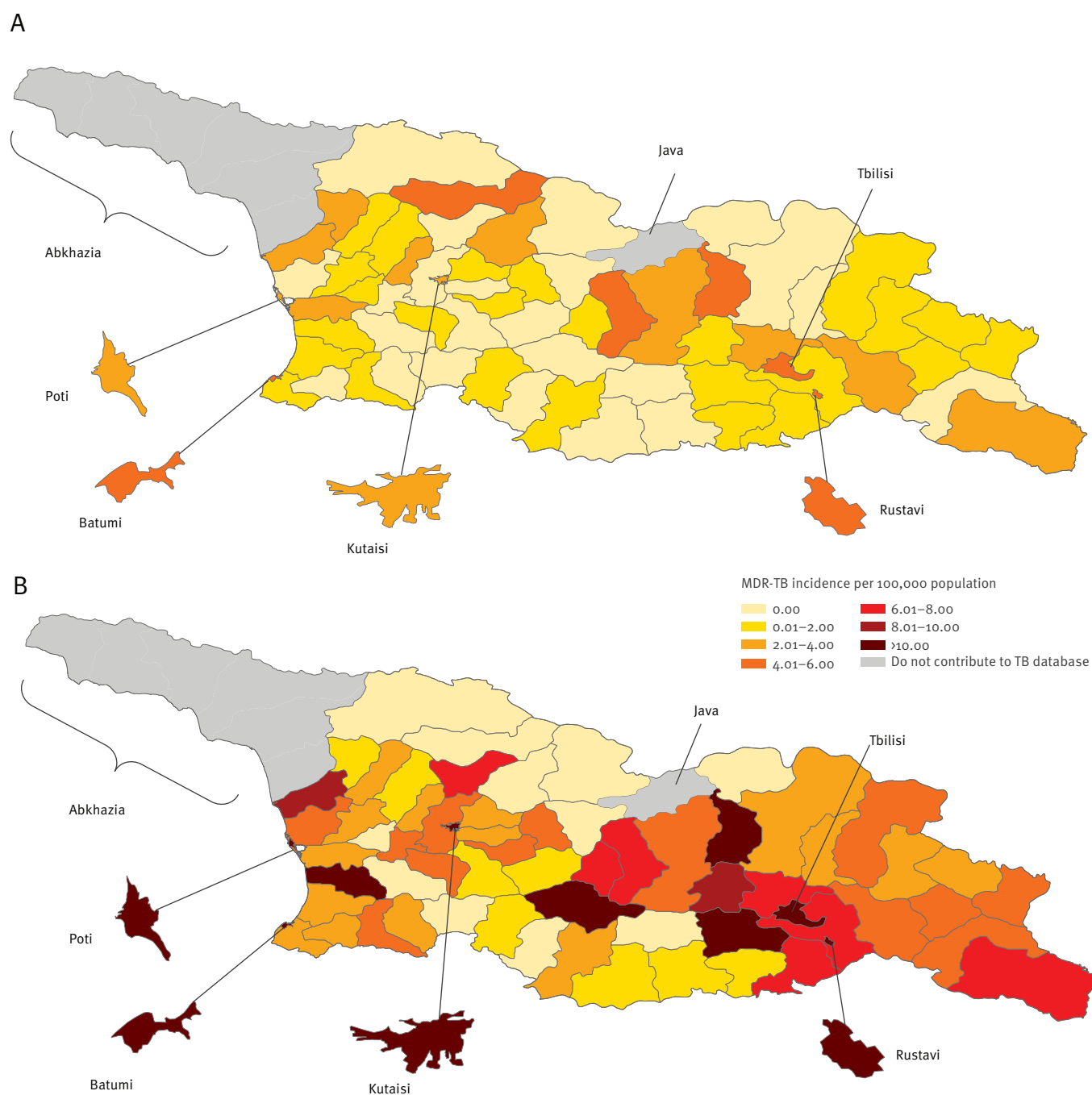
Both the MDR-TB incidence per 100,000 and the percentage of TB cases with MDR-TB were higher in urban areas than in rural areas. MDR-TB incidence in urban areas was 20.7/100,000 compared with 6.6/100,000 in rural areas and the percentage of TB cases with MDR-TB in urban areas was 17.6% compared with 12.1% in rural areas (Table 1). In particular, the percentage of new TB cases with MDR-TB in urban areas was 13.5% as compared to 7.5% in rural areas. Closer examination of the data in individual cities indicates that the largest cities, Tbilisi and Kutaisi, are the main drivers of this phenomenon (Figure 3). We note that in those districts with higher point estimates of MDR-TB risk than these two cities, the estimates are based on small sample sizes and so have quite limited precision (Figure 3).

### Individual-level factors associated with multidrug-resistant tuberculosis among tuberculosis cases

Among new TB cases, living in a city ('urban area', Table 3) was statistically significantly associated with being diagnosed with MDR-TB (odds ratio (OR): 1.43; 95% CI: 1.20–1.72;  $p < 0.001$ ) compared with living in a rural area. New TB cases aged 35 years and over were

**FIGURE 1**

Average annual incidence of notified multidrug-resistant TB per 100,000 population among new (A) and previously treated (B) TB cases by district/city, Georgia, January 2009–June 2011



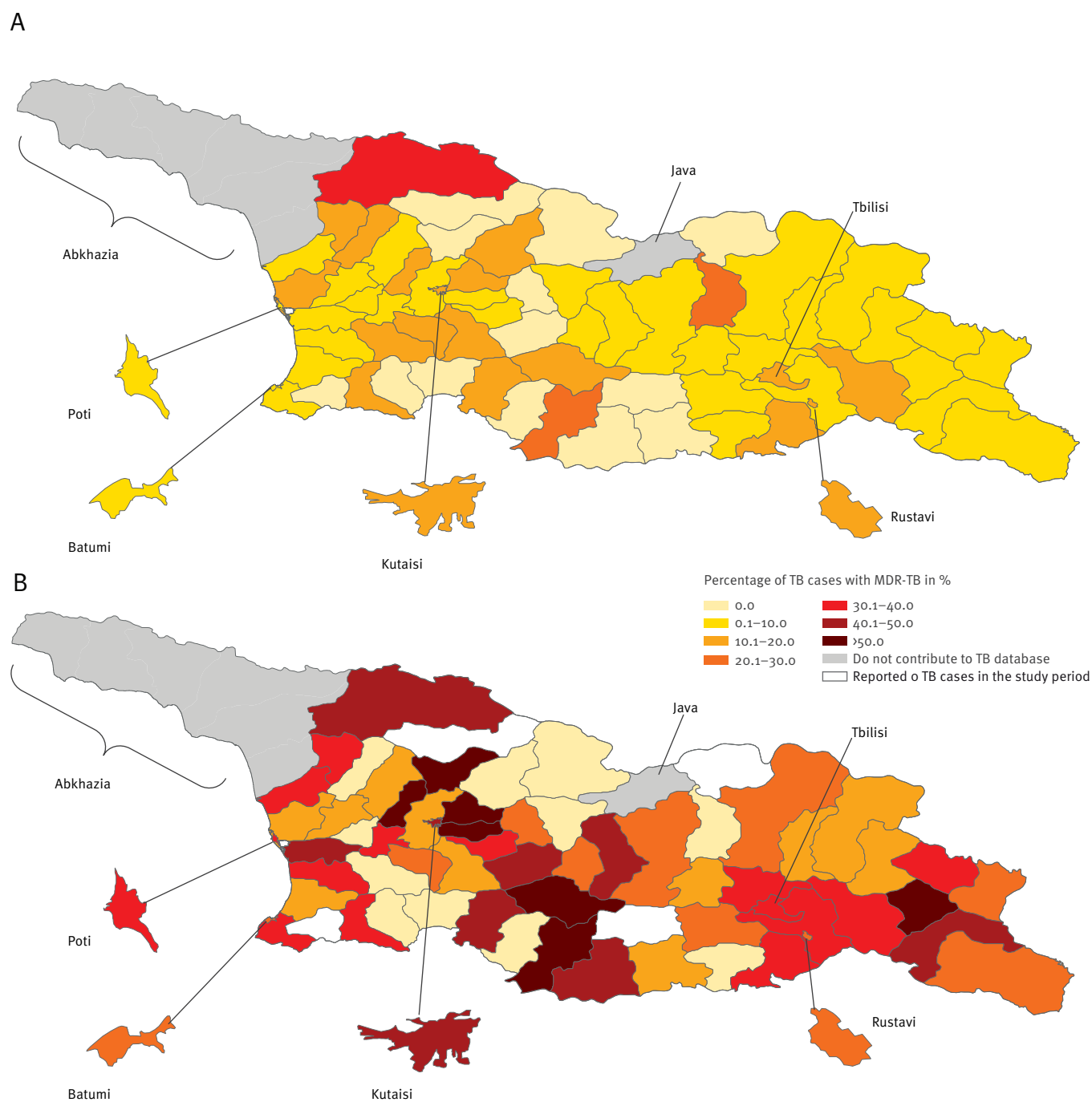
MDR-TB: multidrug-resistant tuberculosis; TB: tuberculosis.

The boundaries of the districts and cities as defined in the National Census [4] are shown. The five areas listed as cities in the National Census are labelled by name and are not shaded in grey. Two areas of Georgia do not report TB cases to the databases analysed: the region of Abkhazia and the district of Java. These two areas are labelled by name and shaded in grey.



**FIGURE 2**

Risk of multidrug-resistant tuberculosis (MDR-TB), as percentage of tuberculosis cases with MDR-TB among new (A) and previously treated tuberculosis cases (B) by district or city, Georgia, January 2009–June 2011

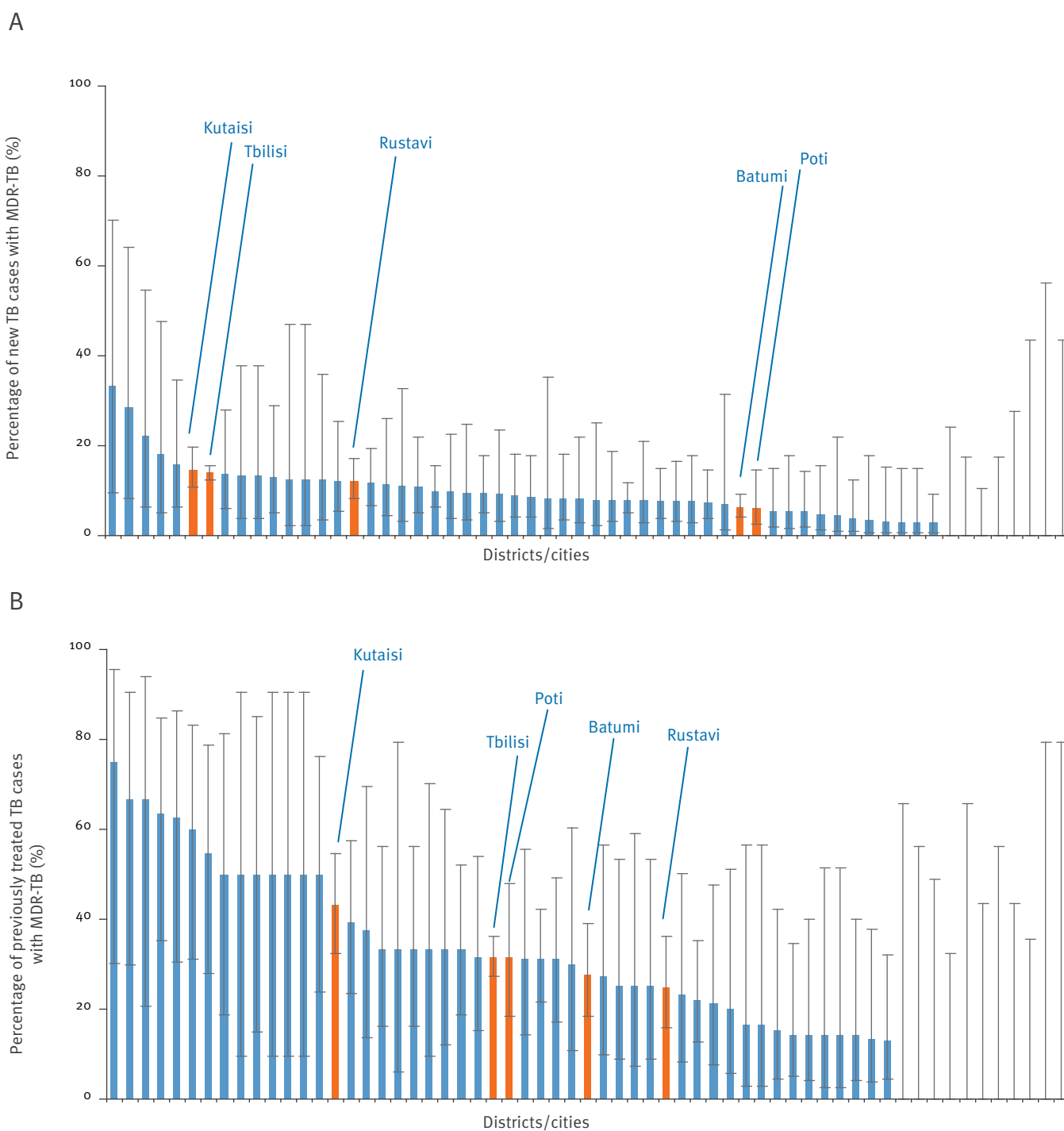


TB: tuberculosis.

The boundaries of the districts and cities as defined in the National Census [4] are shown. The five areas listed as cities in the National Census are labelled by name and are not shaded in grey. Two areas of Georgia do not report TB cases to the databases analysed: the region of Abkhazia and the district of Java. These two areas are labelled by name and shaded in grey.

**FIGURE 3**

Percentage of notified new (A) and previously treated (B) tuberculosis cases with multidrug-resistant tuberculosis confirmed through drug susceptibility testing by district/city in Georgia, January 2009–June 2011



MDR-TB: multidrug-resistant tuberculosis; TB: tuberculosis.

Each bar represents a different district or city and districts/cities are only included if they reported at least one TB case in the study period. Cities (as defined in the National Census [4]) are shown in red and labelled by name; all other districts are shown in blue. Exact binomial confidence intervals are displayed for each district/city.

**TABLE 3**

Individual-level risk factors for multidrug-resistant tuberculosis diagnosis in new and previously treated tuberculosis cases in Georgia, January 2009–June 2011

Variable	New TB cases				Previously treated TB cases			
	Univariable model results		Multivariable model results		Univariable model results		Multivariable model results	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
<b>Demographic and socio-economic factors</b>								
<b>Sex</b>								
Male	Reference	–	NI	NI	Reference	–	Reference	–
Female	1.02 (0.86–1.21)	0.81	NI	NI	0.85 (0.68–1.07)	0.14	0.93 (0.73–1.18)	0.53
<b>Age (years)</b>								
0–34	Reference	–	Reference	–	Reference	–	Reference	–
≥35	0.73 (0.63–0.84)	0.004	0.72 (0.62–0.84)	<0.001	0.94 (0.82–1.08)	0.37	1.09 (0.92–1.29)	0.33
<b>Occupation</b>								
Unemployed	Reference	–	NI	NI	Reference	–	NI	NI
Military	0.34 (0.05–2.34)	0.89	NI	NI	Small numbers	–	NI	NI
Employed	0.98 (0.77–1.25)	0.27	NI	NI	0.96 (0.72–1.27)	0.76	NI	NI
<b>Location at diagnosis</b>								
Rural area	Reference	–	Ref	–	Reference	–	NI	NI
Urban area	1.43 (1.20–1.70)	<0.001	1.43 (1.20–1.72)	<0.001	0.92 (0.77–1.08)	0.30	NI	NI
Penitentiary system	1.22 (0.98–1.52)	0.074	1.20 (0.96–1.50)	0.11	1.19 (1.01–1.40)	0.038	NI	NI
<b>Previously in detention</b>								
No	Reference	–	NI	NI	Reference	–	Reference	–
Yes	0.80 (0.60–1.07)	0.14	NI	NI	1.23 (1.03–1.46)	0.022	1.19 (0.99–1.43)	0.071
<b>Internally displaced person</b>								
No	Reference	–	NI	NI	Reference	–	NI	NI
Yes	0.86 (0.55–1.35)	0.50	NI	NI	0.87 (0.57–1.32)	0.49	NI	NI
<b>TB-related factors</b>								
<b>Disease location</b>								
Pulmonary	Reference	–	NI	NI	Reference	–	NI	NI
Extra-pulmonary	1.04 (0.77–1.41)	0.79	NI	NI	1.11 (0.74–1.67)	0.63	NI	NI
<b>Smear microscopy result</b>								
Negative	Reference	–	Reference	–	Reference	–	NI	NI
Positive	1.15 (0.94–1.42)	0.18	1.21 (0.98–1.49)	0.081	1.01 (0.83–1.23)	0.94	NI	NI
Not tested	2.85 (1.39–5.82)	0.004	2.91 (1.44–5.90)	0.003	1.56 (0.70–3.48)	0.28	NI	NI
<b>Previous treatment outcome</b>								
Cure	NA	NA	NA	NA	Reference	–	NI	NI
Completed treatment	NA	NA	NA	NA	0.92 (0.75–1.13)	0.42	NI	NI
Default	NA	NA	NA	NA	0.59 (0.46–0.75)	<0.001	NI	NI
Failure	NA	NA	NA	NA	1.45 (1.12–1.87)	0.005	NI	NI
Unknown	NA	NA	NA	NA	0.82 (0.57–1.17)	0.27	NI	NI

CI: confidence interval; NA: not applicable; NI: not included in the multivariable model; TB: tuberculosis.

Cases notified between January 2009 and June 2011 are included.

at a lower risk of MDR-TB (OR: 0.72; 95% CI: 0.62–0.84;  $p<0.001$ ). Among previously treated TB cases, those that had previously been in detention may be at a higher risk of MDR-TB (OR: 1.19; 95% CI: 0.99–1.43;  $p=0.071$ ).

## Discussion

We found marked geographical heterogeneity in both MDR-TB incidence and risk. Identification of such ‘hot-spots’ of MDR-TB disease is important in order to appropriately allocate resources and to identify locations for further studies that might inform interventions aimed at reducing the MDR-TB incidence in

Georgia. The variation in both the incidence of MDR-TB per 100,000 population and the percentage of TB cases with MDR-TB between districts was considerable with some districts reporting a disease incidence or risk several times higher than that of other districts (up to 23-fold for MDR-TB incidence and up to 12-fold for MDR-TB risk). This adds further evidence to the literature indicating that such MDR-TB 'hot-spots' may be common to high burden countries [6,12].

Most notably, we found that the risk of MDR-TB among new TB cases was higher in the cities than in rural areas even when adjusting for other potentially confounding factors such as age. Since new TB cases are those that have received either no (or at most one month of) TB treatment previously, the new TB cases with MDR-TB are likely to have been infected with resistant strains. Thus, these results suggest that these urban areas, especially the two largest cities, are where the risk of transmitted MDR-TB is relatively high compared with other less-resistant forms of TB (Table 1). Our results are consistent with a previous study in Georgia that found that living in Tbilisi (the capital city) was a risk factor for MDR-TB [13]. It should be noted that the definitions of urban and rural may overlap and areas on the outskirts of cities may be more similar to rural areas in many respects than they are to the centres of cities.

We found that 10% and 33% of new and previously treated TB cases respectively had MDR-TB. These estimates are nearly identical to those that Georgia reported to the WHO for 2011 although they are slightly lower than estimates reported in previous studies [1,13,14]. These percentages are worrying and within the range that has been reported from other countries of the eastern part of the WHO European Region [3].

Notified MDR-TB incidence in the penitentiary system was 837 per 100,000 people. This is worryingly high and around 50 times the rate estimated for the nationwide population. Young men are overrepresented in prison populations and because this demographic group is likely at greater than average risk of TB and MDR-TB regardless of incarceration status, it is not surprising that MDR-TB is concentrated in prisons. However, even if we assume that the penitentiary system is composed entirely of men aged between 25 and 34 years, we would still only expect a notified MDR-TB incidence of 56/100,000 (Table 2) if the rates of MDR-TB were similar to that of the civilian sector. This is only one fifteenth of the rate that we actually found in the prisons. It is also possible that screening among prisoners at time of entry could partially account for the increased notifications in this setting. However, since only around 10% of TB cases diagnosed in prison are found at entry (M. Gegia, personal communication, 14 May 2013) we think entry screening is unlikely to account for the entire excess burden observed in the prisons. High MDR-TB incidence in the penitentiary system of the order of magnitude found in this study has also been identified previously in Georgia [15] and

in Moldova [6] (also in the eastern part of the WHO European Region). The percentage of new TB cases with MDR-TB in the penitentiary system is nearly identical to that in the rest of the country indicating that the high MDR-TB incidence in that setting is driven by increased TB risk rather than a specific risk of transmitted MDR-TB. However, increased MDR-TB risk among TB cases in the penitentiary system has been found previously within Georgia and elsewhere in the eastern part of the WHO European Region, for example, in Russia and Ukraine [16-18].

Potential limitations of our study include those commonly found when using routinely collected surveillance data. Theoretically, the identification of substantial geographical heterogeneity in MDR-TB incidence in a setting may be attributable to variable case finding and reporting of TB and variable culture and DST use in MDR-TB diagnosis. However, Georgia is a small country with a relatively well-structured TB diagnosis and testing system; variability in case detection across the country is likely to be minimal and culture and DST are respectively required (under national policy) for all TB suspects and culture positive cases. Despite the completeness of this dataset compared to the majority of TB surveillance datasets from high burden countries, bias was potentially introduced by the absence of one third of culture and DST results from the TB notifications database. However, examination of the percentage of missing data from subgroups in the data indicated that there was no systematic bias that would have substantially affected our results. For example, data on culture results were missing from 32% and 33% of urban and rural cases respectively, 32% and 39% from male and female cases respectively and between 30% and 39% from each age class, other than those under 15 years where we would expect greater percentages missing due to difficulties with obtaining specimens. In this study, we attributed each case to a district; we recommend that future studies be conducted that allow the collection of global positioning system (GPS) coordinates of residential locations to allow for more refined spatial analysis, particularly in urban settings.

Aside from a marked difference between urban and rural areas, the underlying reasons for the spatial heterogeneity in MDR-TB are unclear. We attempted to obtain further information on potential explanatory factors such as average household income or proportion of the population in a district that are unemployed from the National Census. However, these data were only available at the national level rather than the district level and without data for each district, we could not investigate associated factors statistically. Understanding these patterns of spread of MDR-TB, which appear to be most severe within urban settings and penitentiaries, requires more refined geographical and genotypic data. Our analysis, which is limited to routinely available data has highlighted important

heterogeneities but leaves many questions about the causes of this variability unanswered.

We have identified substantial geographic heterogeneity in both the risk and incidence of MDR-TB in Georgia, a pattern that may be commonly found in countries in the eastern part of the WHO European Region. Such analysis is crucial to determine where to target resources and prioritise areas for further studies and interventions aimed at reducing the considerable MDR-TB prevalence in these high burden countries.

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## Conflict of interest

None declared.

## Authors' contributions

HEJ and TCo conceived and designed the study. UN and TCh collected the data. HEJ analysed the data. MG, JF and IK helped interpret the results. HEJ and TCo wrote the first draft of the manuscript. All authors contributed to revising the manuscript and read and approved the final version.

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# Laboratory diagnosis of paediatric tuberculosis in the European Union/European Economic Area: analysis of routine laboratory data, 2007 to 2011

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Laboratory confirmation of paediatric tuberculosis (TB) is frequently lacking. We reviewed the range of routine laboratory tests and their performance in different biological samples used to diagnose active TB in children. A questionnaire-based survey was conducted among the European Reference Laboratory Network for TB followed by collection of routine laboratory data on 10,549 paediatric samples tested in 2007 to 2011 at six reference laboratories (in Croatia, Germany, Italy, Latvia, Lithuania and the United Kingdom (UK)). The questionnaire showed that all laboratories used rapid assays. Non-respiratory samples were collected more often in Germany (135/275, 49.1%) and the UK (490/2,140, 22.9%) compared with Croatia (138/2,792, 4.9%), Latvia (222/2,401, 9.2%) and Lithuania (76/1,549, 4.9%). Overall laboratory positivity rates (isolation of *Mycobacterium tuberculosis* complex and/or identification of its nucleic acids in a sample) were higher in lymph node and gastric aspirate samples (14/203 (6.9%) and 43/1,231 (3.5%)) than in sputum samples (89/4,684 (1.9%)). Pooled sensitivity, specificity, positive and negative predictive values and accuracy of molecular assays assessed against solid or liquid culture were 79.2%, 93.6%, 67.1%, 96.5% and 91.6%, respectively. A more intensive approach in obtaining gastric aspirate and non-respiratory samples may increase laboratory confirmation of paediatric TB. Major effort is needed in optimisation and validation of molecular tests in these samples.

## Introduction

Tuberculosis (TB) affects globally about 490,000 children under 15 years-old, with 64,000 related deaths

occurring every year [1]. Paediatric TB is an indicator of recent transmission in the population. Often, children experience more severe forms of the disease, such as miliary or meningeal TB [2,3]. Young children are rarely able to expectorate sputum; therefore, other respiratory samples, such as gastric aspirates (GA) or bronchoalveolar lavages (BAL), can be obtained for diagnostic purposes, although these procedures are more unpleasant for a child than sputum collection [1,3-5]. Collection of non-respiratory samples (lymph node (LN), pus or tissue biopsy) is necessary to diagnose extrapulmonary TB; however, these procedures are relatively invasive [6,7].

TB in children often has a paucibacillar nature, resulting in microscopy smears that are negative [3,4,8]. Culture isolation, a more sensitive method, takes up to 14 days due to slow growth of *Mycobacterium tuberculosis* complex (MTB(C)) bacteria; however, even culture is seldom positive in paediatric specimens due to very few bacilli present in a sample [3,4,8]. Further full drug susceptibility testing (DST) can only be done once an isolate is available and takes two more weeks [9,10].

Consequently, the diagnosis of paediatric TB often relies on a combination of clinical judgment and radiological findings, prompting initiation of treatment without clear laboratory evidence [1,8,9,11,12]. Unlike laboratory criteria, there is as yet no universally applied diagnostic algorithm based on clinical and radiological criteria that are objective [8,11,12].

The most important advance in TB diagnosis is the introduction of the internationally endorsed molecular assay Xpert MTB/RIF (Cepheid Inc., Sunnyvale, CA, United States), which identifies MTB and rifampicin resistance from sputum samples [13]. A growing body of evidence supports the adequacy of Xpert MTB/RIF for adult TB diagnosis [14–16].

Recent studies have investigated the performance of the Xpert MTB/RIF system in children and showed promising results when the test is applied to sputum, nasopharyngeal aspirates (NPA), GA or non-respiratory samples [4,6,7,17–19]. However, there are limited data on the usefulness of molecular assays for paediatric TB diagnosis in Europe, where TB prevalence is low [20].

The aim of the study was to give an overview of the range of routine diagnostic tests and their performance for different types of samples used to diagnose active TB in children across European laboratories.

## Methods

### Study design

The study was planned in two stages. Firstly, we invited all (38) national TB reference laboratories across the European Union (EU)/European Economic Area (EEA) countries that are members of the European Reference Laboratory Network for TB (ERLN-TB) – an initiative supported by the European Centre for Disease Prevention and Control (ECDC) [21] – to participate in a questionnaire-based survey. The questionnaire aimed to determine the variety of algorithms used to diagnose paediatric TB and the type of samples collected from children; it contained 53 questions and was based on an earlier questionnaire used across the ERLN-TB [22].

Secondly, six laboratories of the network (referred to hereafter as study sites) agreed to provide their routine data for all consecutive primary samples and reference cultures referred for diagnosis from children younger than 15 years-old with suspected TB during 2007 to 2011.

Primary samples were defined as specimens referred to a reference laboratory for primary diagnostics, while reference cultures were cultures isolated by local laboratories and referred to a reference laboratory for confirmation, DST or molecular typing.

Low-incidence western and central European settings were represented by the following laboratories: the National Reference Centre for Mycobacteria at Forschungszentrum Borstel, Borstel, Germany; San Raffaele Scientific Institute, in collaboration with the Institute ‘Villa Marelli’, Niguarda Ca’ Granda Hospital, Milan, Italy; the National Mycobacterium Reference Laboratory, Public Health England, London, United Kingdom (UK) and the National Mycobacterium Reference Laboratory, National Institute of Public Health, Zagreb, Croatia (TB incidence in 2012 being

5.6, 6.7, 15.0 and 14.0 cases per 100,000 population in the respective countries [23]).

The National TB Reference Laboratory at the Latvian Infectology Centre, Upeslejas, Latvia, and the Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital, Vilnius, Lithuania, represented medium-incidence eastern European countries, with TB incidence in 2012 being 53.0 and 66.0 cases per 100,000 population, respectively [23].

Only partial data were available from the Italian site for 2011; this is the only site that does not have a national reference laboratory function, covering mainly the Lombardy region of Italy. Germany provided data for 2011 only. We did not exclude control samples, as there were only a few of those.

The laboratory data were collected using an Excel-based tool, with line-listing by sample. The Croatian, German, Italian and UK sites received a small proportion of all paediatric samples in the country (estimated by the laboratory directors as less than 10%), while the Lithuanian site covered approximately half and the Latvian site all paediatric samples in their countries.

Laboratory positivity was confirmed by a positive culture (on solid or liquid media) identified as MTB(C) or non-tuberculous mycobacteria (NTM) and/or by the identification of MTB(C) or NTM nucleic acid directly in a sample by a molecular assay.

### Ethics statement

Routine laboratory data were sent to the Robert Koch Institute, Berlin, Germany, without personal identifiers. The study obtained a waiver of informed consent and ethics review permission from the Robert Koch Institute.

### Data analysis

Per-sample and per-patient analyses were conducted. Samples were categorised as ‘respiratory’ (sputum, BAL, GA, pleural fluid and other respiratory samples such as NPA), ‘non-respiratory’ (cerebrospinal fluid (CSF), LN, pus, blood, other tissue biopsy, urine and other non-respiratory samples such as pericardial fluid) and ‘unknown’ (where exact information on sample type was missing from the records).

Calculations related to the MTB(C) did not include the *Mycobacterium bovis* strain used in the Bacillus Calmette–Guérin (BCG) vaccine.

The incremental positivity rate, defined as additional sensitivity gained by testing more than one sample from the same patient, was calculated for GA samples.

Associations between correct diagnostic yield and sample type were tested using Pearson’s chi-squared test or Fisher’s exact test. Statistical tests were two-sided at  $\alpha=0.05$ .

**TABLE 1**

Laboratory diagnosis of paediatric tuberculosis: types of paediatric primary samples received across the six study sites, 2007–2011 (n=9,157)

Site	Total number of samples received	Sample type		
		Respiratory <sup>a</sup> n (%)	Non-respiratory <sup>a</sup> n (%)	Unknown n (%)
Croatia CIPH	2,792	2,650 (94.9)	138 (4.9)	4 (0.1)
Germany <sup>b</sup> NRCM	275	129 (46.9)	135 (49.1)	11 (4.0)
Italy <sup>b</sup> HSR	340	NA	NA	NA
Latvia NTRL	2,401	2,167 (90.3)	222 (9.2)	12 (0.5)
Lithuania TBL	1,549	1,464 (94.5)	76 (4.9)	9 (0.6)
United Kingdom NMRL	2,140	1,643 (76.8)	490 (22.9)	7 (0.3)
<b>All sites</b>	<b>9,157<sup>c</sup></b>	<b>8,053 (87.9)</b>	<b>1,061 (11.6)</b>	<b>43 (0.5)</b>

CIPH: Croatian National Institute of Public Health, National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', Niguarda Ca' Granda Hospital; NA: data not available; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

<sup>a</sup> Respiratory samples: sputum, gastric aspirate, bronchoalveolar lavage, pleural fluid, other respiratory samples (e.g. nasopharyngeal aspirate). Non-respiratory samples: cerebrospinal fluid, lymph node, pus, other biopsy tissue, urine, other non-respiratory samples (e.g. pericardial fluid).

<sup>b</sup> Data from 2011 only.

<sup>c</sup> Does not include the 340 samples from Italy.

The results of the different types of molecular tests were pooled since the number of samples did not allow stratified analysis of each test. Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) and the accuracy of the pooled molecular assays was assessed against culture and DST results in samples tested by both methods.

The answers from the survey questionnaire were entered into EpiData (EpiData Association, Odense, Denmark) and analysed using STATA (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). The routine laboratory data were collected from the study sites using a Microsoft Excel spreadsheet and analysed in STATA.

## Results

### Questionnaire-based survey

A total of 21 TB reference laboratories from Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Italy (two laboratories), Latvia, Lithuania, Norway, Portugal, Slovenia, Spain (two laboratories), Sweden and the UK sent their responses.

The population served by these laboratories varied between 400,000 and 20,000,000 inhabitants. Annually, the laboratories receive a median of 70

(range: 6–950) paediatric primary samples and reference cultures representing a median of 3.1% (range: 0.3–8.3%) of the total laboratory workload.

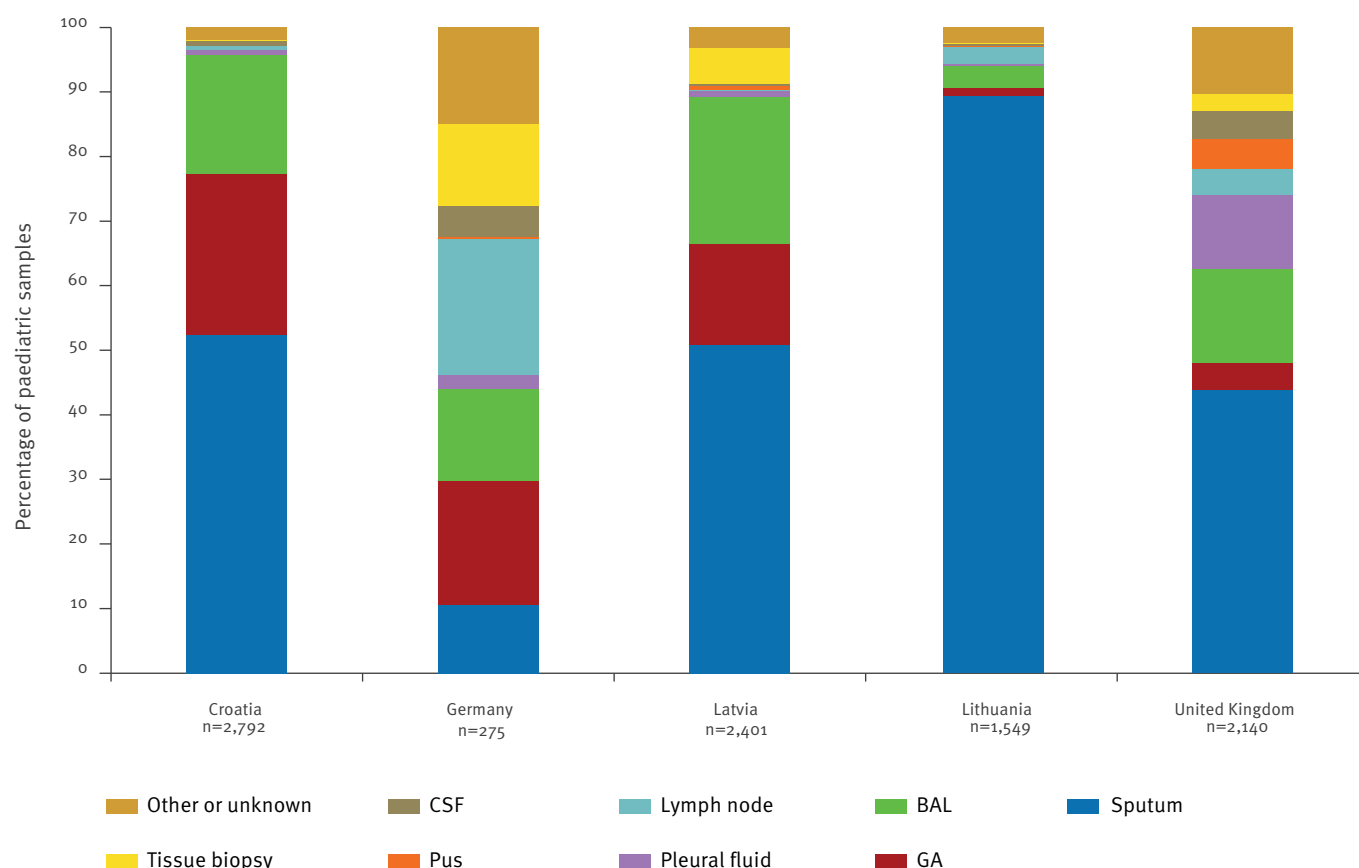
Of the 21 laboratories, 16 receive primary samples; the Latvian and Lithuanian laboratories receive primary samples only. Five laboratories receive reference cultures only (Belgium, Czech Republic, Finland, Norway and Spain). The most common sample type received from children is sputum (16 laboratories), followed by GA and BAL (15 laboratories each). A total of 11 laboratories reported receiving more than one type of sample from each child.

All laboratories receiving primary samples perform smear microscopy, molecular identification, culture (solid or liquid media, commercial or in-house) and first- and second-line DST. All undergo international quality assurance, demonstrating satisfactory performance.

The most frequently used direct rapid molecular identification assay included Xpert MTB/RIF (n=10) and GenoType MTBDR<sub>plus</sub> (Hain Lifescience, GmbH, Germany) (n=8); 10 laboratories used more than one assay. All laboratories rapidly identified MTB(C) from positive culture using GenoType CM/AS (Hain Lifescience, GmbH, Germany) (n=11), BD MGIT TBc Id (Becton Dickinson, United States) and GenoType MTBDR<sub>plus</sub> assays (n=8 each). Nine laboratories

## FIGURE

Laboratory diagnosis of paediatric tuberculosis: type and percentage of paediatric primary samples received across five study sites, 2007–2011 (n=9,157)



CSF: cerebrospinal fluid; BAL: bronchoalveolar lavage; GA: gastric aspirate.

Data from the Italian laboratory were not included, since available data specified only that the samples were either respiratory or non-respiratory, without further information on specific sample type.

routinely used GenoType MTBDRsl (Hain Lifescience, Germany) for rapid second-line DST.

In 17 laboratories, the algorithms used to diagnose paediatric and adult TB did not differ; four laboratories reported the use of extra tests for children. In Latvia and Lithuania, for example, when there was a positive culture from a biopsy sample from a child GenoType MTBC (Hain Lifescience, Germany) was used to distinguish between MTB and *M. bovis* BCG.

## Routine laboratory data

### Number and range of primary samples received

A total of 9,157 primary samples and 1,392 reference cultures were received from children across all six study sites within the five-year study period (Table 1).

Of all primary samples, the vast majority (8,053/9,157, 87.9%) were respiratory, including 4,974 sputum (61.8%), 1,467 BAL (18.2%), 1,232 GA (15.3%) and 298 pleural fluid (3.7%) samples; 1,061 samples were

non-respiratory, including 230 tissue biopsy samples other than LN (21.7%), 205 LN (19.3%), 182 urine (17.2%), 140 CSF (13.2%) and 118 pus (11.1%) (Figure).

In the Croatian, Latvian and Lithuanian sites, less than 10% of all paediatric samples were non-respiratory (138/2,792, 4.9%; 222/2,401, 9.2%; 76/1,549, 4.9%, respectively), whereas in the UK site, this proportion was higher, 490/2,140 (22.9%). The German site received more non-respiratory than respiratory primary samples (135/275, 49.1% and 129/275, 46.9%, respectively), with LN being the most common sample type (58/275, 21.1%) (Table 1, Figure).

### Microscopy, culture and drug susceptibility testing

Most of the primary samples (8,176/9,157, 89.3%) were subjected to smear microscopy, resulting in a 3.3% (268/8,176) positivity rate (Table 2). More non-respiratory than respiratory samples were positive by microscopy (82/816, 10.0% vs 181/7,320, 2.5%,  $p<0.0001$ ).

TABLE 2

Laboratory diagnosis of paediatric tuberculosis: tests performed on paediatric primary samples across the six study sites, 2007–2011 (n=9,157)

Type of laboratory test	Result	Sample type		All samples <sup>b</sup>
		Respiratory <sup>a</sup> n (%)	Non-respiratory <sup>a</sup> n (%)	n (%)
Smear microscopy	All	7,320 (100)	816 (100)	8,176 (100)
	Positive	181 (2.5)	82 (10.0)	268 (3.3)
	Negative	7,139 (97.5)	734 (90.0)	7,908 (96.7)
Culture <sup>c</sup>	All	7,749 (100)	1,015 (100)	8,806 (100)
	Positive <sup>d</sup>	392 (5.1)	142 (14.0)	541 (6.1)
	Positive: MTB(C) <sup>e</sup>	149 (1.9)	40 (3.9)	195 (2.2)
	Positive: NTM	211 (2.7)	89 (8.8)	314 (3.6)
	Negative	7,213 (93.1)	848 (83.5)	8,096 (91.9)
	Contaminated	144 (1.8)	25 (2.5)	169 (1.9)
	Indeterminate	2 (0.6)	12 (4.7)	14 (2.4)
Direct rapid molecular identification of MTB(C) in primary samples <sup>f</sup>	All	308 (100)	258 (100)	578 (100)
	Positive for MTB(C) <sup>e</sup>	64 (20.8)	28 (10.8)	93 (16.1)
	Negative	242 (78.6)	218 (84.5)	471 (81.5)
	Indeterminate	2 (0.6)	12 (4.7)	14 (2.4)
Direct rapid molecular DST on primary samples	All	64 (100)	28 (100)	93 (100)
	Rifampicin susceptible	59 (92.2)	26 (92.9)	85 (91.4)
	Rifampicin resistant	4 (6.2)	2 (7.1)	7 (7.5)
	Indeterminate	1 (1.6)	0 (0.0)	1 (1.1)

BCG: *Bacillus Calmette–Guérin*; DST: drug susceptibility testing; MTB(C): *Mycobacterium tuberculosis* complex; NTM: non-tuberculous mycobacteria.

<sup>a</sup> Respiratory samples: sputum, gastric aspirate, bronchoalveolar lavage, pleural fluid, other respiratory samples (e.g. nasopharyngeal aspirate); non-respiratory samples: cerebrospinal fluid, lymph node, pus, other biopsy tissue, urine, other non-respiratory samples (e.g. pericardial fluid).

<sup>b</sup> Including samples of unknown type.

<sup>c</sup> Either Löwenstein–Jensen, Mycobacteria Growth Indicator Tube or other solid or liquid media. Only one positive culture result per sample was analysed.

<sup>d</sup> 32 of the 541 positive culture samples were either not further identified or were identified as *M. bovis* BCG.

<sup>e</sup> *M. bovis* BCG not included.

<sup>f</sup> Used the following assays: Croatia: GeneXpert MTB/RIF, Amplified MTB Direct Test GenProbe; Germany: GeneXpert MTB/RIF, GenoType MTBDR<sub>plus</sub>, in-house test; Italy: GeneXpert MTB/RIF, INNO-LiPA; Latvia: GeneXpert MTB/RIF; Lithuania: GeneXpert MTB/RIF; United Kingdom: GeneXpert MTB/RIF, GenoType MTBDR<sub>plus</sub>, INNO-LiPA, IS6110 sequencing.

Almost all (8,806/9,157, 96.2%) samples were cultured and a positive culture was obtained in 541 (6.1%) of the 8,806 primary samples. Culture was more often positive from non-respiratory than from respiratory samples (142/1,015, 14.0% vs 392/7,749, 5.1%,  $p<0.0001$  for all positive cultures; 40/1,015, 3.9% vs 149/7,749, 1.9%,  $p=0.0001$  for MTB(C)-positive cultures) (Table 2).

Across all sites, the culture contamination rate (on solid and liquid media) was 169/8,806 (1.9%) (Table 2), ranging from 0/2,884 (0%) at the Croatian to 16/240 (6.7%) at the German site.

Per-sample analysis showed that MTB(C) was detected in 236/8,851 (2.7%) of primary samples with the highest rates at the German and UK sites, 23/275 (8.4%) and 107/2,132 (5.0%), respectively (Table 3). The sample positivity rates for MTB(C) were significantly higher

in pus ( $p=0.0005$ ), LN ( $p<0.0001$ ), CSF ( $p=0.002$ ) and other tissue biopsy ( $p=0.002$ ) samples compared with sputum samples. Among respiratory samples, MTB(C) was more commonly detected in GA samples than in sputum samples (43/1,231, 3.5% vs 89/4,684, 1.9%,  $p=0.002$ ). No statistically significant difference between MTB(C) detection rates in GA or BAL samples was observed. Per-patient analysis showed that MTB(C) was isolated in 156/5,156 (3.0%) of children (Table 4). MTB(C) was detected in 31/643 (4.8%) patients who submitted GA samples; of them, 21/31 patients submitted at least two GA samples. The first GA sample identified 16 positive patients, the second identified an additional four and the third, another positive patient.

Multidrug resistance (MDR), defined as resistance to at least rifampicin and isoniazid, was seen in 10/156 (6.4%) of patients ranging from 0/23 and 0/4 at Croatian



**TABLE 3**

Laboratory diagnosis of paediatric tuberculosis: paediatric primary samples identified as MTB(C) across five study sites, 2007–2011 (n=236)

Site	Number of samples identified as MTB(C) <sup>a</sup> /total number per sample type (%)								
	Sputum	GA	BAL	Lymph node	Pus	CSF	Tissue biopsy	Other samples	All samples
Croatia CIPH	6/1,402 (0.4)	20/694 (2.9)	9/516 (1.7)	1/20 (5.0)	0/0 (0.0)	0/19 (0.0)	0/3 (0.0)	1/138 (0.7)	<b>37/2,792 (1.3)</b>
Germany <sup>b</sup> NRCM	1/29 (3.4)	9/53 (17.0)	5/39 (12.8)	2/58 (3.4)	0/1 (0.0)	1/13 (7.7)	2/35 (5.7)	3/47 (6.4)	<b>46/2,397 (1.9)</b>
Latvia NTRL	16/1,219 (1.3)	4/378 (1.1)	13/546 (2.4)	1/2 (50.0)	1/10 (10.0)	1/9 (11.1)	6/135 (4.4)	4/98 (4.1)	<b>107/2,132 (5.0)</b>
Lithuania TBL	13/1,099 (1.2)	0/17 (0.0)	2/54 (3.7)	4/40 (10.0)	0/1 (0.0)	0/6 (0.0)	1/1 (100)	3/37 (8.1)	<b>23/1,255 (1.8)</b>
United Kingdom NMRL	53/935 (5.7)	10/89 (11.2)	7/312 (2.2)	6/83 (7.2)	8/102 (7.8)	7/91 (7.7)	6/56 (10.7)	10/464 (2.2)	<b>107/2,132 (5.0)</b>
<b>All sites</b>	<b>89/4,684 (1.9)</b>	<b>43/1,231 (3.5)</b>	<b>36/1,467 (2.5)</b>	<b>14/203 (6.9)</b>	<b>9/114 (7.9)</b>	<b>9/138 (6.5)</b>	<b>15/230 (6.5)</b>	<b>21/784 (2.7)</b>	<b>236/8,851 (2.7)</b>

BAL: bronchoalveolar lavage; BCG: Bacillus Calmette–Guérin; CIPH: Croatian National Institute of Public Health; National Mycobacterium Reference Laboratory; CSF: cerebrospinal fluid; GA: gastric aspirate; MTB(C): *Mycobacterium tuberculosis* complex; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

<sup>a</sup> By isolation of MTB(C) culture or identification of MTB(C) nucleic acid in a sample. *M. bovis* BCG results are not included.

<sup>b</sup> Data from 2011 only.

**TABLE 4**

Laboratory-confirmed paediatric cases<sup>a</sup> across the six study sites (with primary samples only), 2007–2011 (n=156)

Site	Type of primary sample		Identification of MTB(C)	Drug susceptibility testing
	Sputum	Gastric aspirate		
	n samples/ N patients (ratio)	n samples/ N patients (ratio)	n MTB(C)-positive patients <sup>c</sup> /N patients (%)	n with MDR/ N MTB(C)-positive patients <sup>c</sup> (%)
Croatia CIPH	1,402/1,006 (1.4)	694/391 (1.8)	23/1,681 (1.4)	0/23 (0.0)
Germany <sup>b</sup> NRCM	29/21 (1.4)	53/22 (2.4)	7/152 (4.6)	1/7 (14.3)
Italy <sup>b</sup> HSR	NA	NA	4/200 (2.0)	0/4 (0.0)
Latvia NTRL	1,219/504 (2.4)	378/170 (2.2)	40/1,151 (3.5)	3/40 (7.5)
Lithuania TBL	1,385/774 (1.8)	18/13 (1.4)	17/886 (1.9)	3/17 (17.7)
United Kingdom NMRL	939/382 (2.5)	89/47 (1.9)	65/1,086 (6.0)	3/65 (4.6)
<b>All sites</b>	<b>4,974/2,687 (1.9)</b>	<b>1,232/643 (1.9)</b>	<b>156/5,156 (3.0)</b>	<b>10/156 (6.4)</b>

CIPH: Croatian National Institute of Public Health; National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', NiguardaCa' Granda Hospital;MDR: multidrug resistance (resistant to rifampicin and isoniazid); NA: data not available; NMRL: National Mycobacterium Reference Laboratory; MTB(C): *Mycobacterium tuberculosis* complex; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

<sup>a</sup> By isolation of MTB(C) culture or identification of MTB(C) nucleic acid from any of the patient's samples.

<sup>b</sup> Data from 2011 only.

<sup>c</sup> *M. bovis* BCG not included.

**TABLE 5**

Laboratory diagnosis of paediatric tuberculosis: performance of molecular assays compared with culture in detecting MTB(C) in primary samples across the six study sites<sup>a</sup>, 2007–2011

Sample type and smear results	Sensitivity <sup>b</sup>		Specificity <sup>c</sup>		Positive predictive value <sup>d</sup>		Negative predictive value <sup>e</sup>		Accuracy <sup>f</sup>	
	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)
All <sup>g,h</sup>	57/72	79.2 (68.0–87.8)	411/439	93.6 (90.9–95.7)	57/85	67.1 (56.0–76.9)	411/426	96.5 (94.3–98.0)	468/511	91.6 (88.8–93.8)
All smear positives	42/46	91.3 (79.2–97.6)	41/56	73.2 (59.7–84.2)	42/67	62.7 (60.3–84.5)	41/45	91.1 (78.8–97.5)	83/102	81.4 (72.4–88.4)
All smear negatives	11/21	52.4 (29.8–74.3)	287/299	96.0 (93.1–97.9)	11/23	47.8 (26.8–69.4)	287/297	96.6 (93.9–98.4)	298/320	93.1 (89.8–95.6)
Respiratory <sup>g</sup>	48/54	88.9 (77.4–95.8)	224/236	94.9 (91.3–97.3)	48/60	80.0 (67.7–89.2)	224/230	97.4 (94.4–99.0)	272/290	93.8 (90.4–96.3)
Respiratory smear positives	40/42	95.2 (83.8–99.4)	15/20	75.0 (50.9–91.3)	40/45	88.9 (75.9–96.3)	15/17	88.2 (63.6–98.5)	55/62	88.7 (78.1–95.4)
Respiratory smear negatives	5/8	62.5 (24.5–91.5)	161/168	95.8 (91.6–98.3)	5/12	41.7 (15.2–72.3)	161/164	98.2 (94.7–99.6)	166/176	94.3 (89.8–97.2)
Non-respiratory <sup>g</sup>	7/16	43.8 (19.8–70.1)	177/192	92.2 (87.4–95.6)	7/22	31.8 (13.9–54.9)	177/186	95.2 (91.0–97.8)	184/208	88.5 (83.3–92.5)
Non-respiratory smear positives	2/4	50.0 (6.8–93.2)	24/34	70.6 (52.5–84.9)	2/12	16.7 (2.1–48.4)	24/26	92.3 (74.9–99.1)	26/38	68.4 (51.3–82.5)
Non-respiratory smear negatives	5/12	41.7 (15.2–72.3)	118/123	95.9 (90.8–98.7)	5/10	50.0 (18.7–81.3)	118/125	94.4 (88.8–97.7)	123/135	91.1 (85.0–95.3)
Sputum <sup>g</sup>	34/37	91.9 (78.1–98.3)	54/57	94.7 (85.4–98.9)	34/37	91.9 (78.1–98.3)	54/57	94.7 (85.4–98.9)	88/94	93.6 (86.6–97.6)
Sputum smear positives	32/33	97.0 (84.2–99.9)	6/9	66.7 (29.9–92.5)	32/35	91.4 (76.9–98.2)	6/7	85.7 (42.1–99.6)	38/42	90.5 (77.3–97.3)
Sputum smear negatives	1/3	33.3 (0.8–90.6)	48/48	100 (92.6–100)	1/1	100 (2.5–100)	48/50	96.0 (86.3–99.5)	49/51	96.0 (86.5–99.5)
Gastric aspirates <sup>g</sup>	8/10	80.0 (44.4–97.5)	95/99	96.0 (90.0–98.9)	8/12	66.7 (34.9–90.1)	95/97	97.9 (92.7–99.7)	103/109	94.5 (88.4–97.9)
Bronchoalveolar lavage <sup>g</sup>	6/7	85.7 (42.1–99.6)	55/59	93.2 (83.5–98.1)	6/10	60.0 (26.2–87.8)	55/56	98.2 (90.4–100)	61/66	92.4 (83.2–97.5)

CI: confidence interval; MTB(C): *Mycobacterium tuberculosis* complex.

<sup>a</sup> CIPH: Croatian National Institute of Public Health, National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', NiguardaCa Granda Hospital; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

<sup>b</sup> Number of true positives/number of true positives + number of false negatives.

<sup>c</sup> Number of true negatives/number of true negatives + number of false positives.

<sup>d</sup> Number of true positives/number of true positives + number of false positives.

<sup>e</sup> Number of true negatives/number of true negatives + number of false negatives.

<sup>f</sup> Number of true positives + number of true negatives/total number of tested samples.

<sup>g</sup> Number of smear negatives + number of smear positives + number with smear result unknown.

<sup>h</sup> Number of respiratory samples + number of non-respiratory samples + number of samples of unknown type.

and Italian sites to 3/17 of cases at the Lithuanian site (Table 4).

#### Rapid molecular test performance for *Mycobacterium tuberculosis* complex and drug-resistance detection

Routine use of NAAT was initiated in 1996 at the Croatian, in 1994 at the German and Italian, in 2010 at the Latvian, in 2008 at the Lithuanian and in 1999 at the UK sites. A variety of commercial and in-house assays were used on 578/9,157 (6.3%) of samples.

The bacteria in respiratory samples were more often directly identified as MTB(C) than those in non-respiratory samples (64/308, 20.8% vs 28/258, 10.9%,  $p=0.001$ ). The rate of indeterminate results was higher among non-respiratory samples (12/258, 4.7% vs 2/308, 0.6%,  $p=0.0024$ ) (Table 2).

A total of 511 primary samples were tested by both molecular assay and culture for MTB(C) identification. Compared with liquid or solid culture, a rapid molecular test – based on the pooled data analysis – had an overall (for all samples types) sensitivity of

57/72 (79.2%), specificity of 411/439 (93.6%), PPV of 57/85 (67.1%), NPV of 411/426 (96.5%) and accuracy of 468/511 (91.6%) for MTB(C) detection (Table 5). The sensitivity of molecular tests for smear-positive samples vs smear-negative samples was significantly higher (42/46, 91.3% vs. 11/21, 52.4%,  $p=0.0006$ ), while they were less specific (41/56, 73.2% vs 287/299, 96.0%,  $p=0.0001$ ). The lower specificity in the smear-positive samples was due to 12 false-positive results at the UK site tested by INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium) and Xpert MTB/RIF, two at the German site tested by Xpert MTB/RIF and one at Croatian site tested by Xpert MTB/RIF.

The molecular tests detected MTB(C) in 48/54 (88.9%) of respiratory and 7/16 (43.8%) of non-respiratory samples ( $p=0.0004$ ); the specificity was 224/236 (94.9%) and 177/192 (92.2%), respectively. For both respiratory and non-respiratory samples, the sensitivity was again higher for smear-positive than for smear-negative samples; however, the specificity was higher for smear-negative samples for both groups of samples (Table 5).

Molecular assays produced 15/511 (2.9%) of false-negative results and 28/511 (5.5%) of false-positive results for MTB(C) identification; discordant results were related to all types of samples. Mutations coding resistance to rifampicin were detected in 7/93 (7.5%) of all primary samples tested by rapid assays (Table 2). Molecular and phenotypic assays were fully concordant with DST.

#### Non-tuberculous mycobacteria and *Mycobacterium bovis* BCG

Approximately half (989/1,903, 52.0%) of all positive isolates (grown from primary samples and those received as reference cultures) were identified as NTMs at the Croatian (43/94), German (54/90), Italian (26/50) and UK (862/1,564, 55.1%) sites. NTMs were less common at the Latvian (1/67) and Lithuanian (3/38) sites. A proportion of all positive isolates (224/1,903, 11.8%) were identified as *M. bovis* BCG.

NTMs were isolated at the highest rate from LN samples (27/203, 13.3%). NTMs were seen in 136/4,958 (2.7%) of all paediatric patients, with *M. abscessus* as the most frequently identified.

## Discussion

This large study reviewed the analysis of over 10,000 samples tested across a number of European TB reference laboratories and showed the availability and use of all conventional and modern diagnostic techniques across these settings. Although the diagnostic algorithms were similar across laboratories, approaches towards collecting paediatric non-respiratory samples differ between European sites, with very few samples other than sputum obtained from children in Latvia and Lithuania.

Because young children can rarely expectorate but instead swallow their sputum, aspiration of the gastric content is the best (and the least unpleasant) procedure for obtaining a sputum sample suitable for further diagnostics [4,5,19,24]. Our study showed that, compared with sputum samples, testing GA more often results in positive findings. Recent data by Fiebig et al. support this evidence [25]: they report widespread use of GA in Germany and a high (63%) yield of culture and NAAT in paediatric specimens, confirming that it is possible to reliably diagnose TB in children and disagreeing with the common notion of 'difficulties' in laboratory confirmation of paediatric disease.

Our data suggest that collection of several GA samples might help identify further cases, in line with previously published data [5]. Larger studies are needed to assess the differences in diagnostic yield in serial samples.

Extrapulmonary samples also tested positive more frequently than sputum samples did, placing emphasis on the usefulness of collecting non-respiratory materials, in line with some previously published results that showed a good performance of fine-needle aspiration biopsy of LN in confirming TB [26]. Considering the observed small proportion of non-respiratory samples received at the Latvian and Lithuanian sites, it would seem reasonable to expect a low incidence of extrapulmonary TB there. However, crude comparison with World Health Organization (WHO) surveillance data for 2011 showed that while in Croatia, Germany, Italy and the UK extrapulmonary TB represented the minority of paediatric cases (15%, 17%, 19% and 37%, respectively), the majority of notified cases in Latvia and Lithuania were, in contrast, extrapulmonary (59% and 92%, respectively) [23], pointing towards predominantly non-laboratory based diagnosing. Within the framework of our study, infrequent collection of samples other than sputum might be explained by differences in the referral population, with a large number of asymptomatic children being screened for TB as part of contact tracing in medium-incidence settings, such as those of the participating Baltic countries [23]. Relatively low MTB(C)-positivity rates at the Latvian and Lithuanian sites also reflect this screening strategy. There, a lower level of clinical suspicion might be the reason for not carrying out more invasive procedures. Another explanation might be a more cautious attitude of eastern European physicians in Latvia and Lithuania in collecting GA or non-respiratory samples from children, related to the higher invasiveness of these procedures compared with sputum collection, and lack of confidence in the success of laboratory diagnosis. The rates of notified extrapulmonary TB across the EU/EEA vary between 4% and 48% (37.7% in children during 2002 to 2011), possibly reflecting the challenges and differences in diagnosis and confirming the urgent need for improvement [27,28].

Analysis of the performance of the pooled molecular tests showed that the sensitivity was comparable to that reported for the Xpert MTB/RIF in children in other studies: 79.5% for sputum and GA in Vietnam, 90.0 and 68.8% for sputum and GA samples in Zambia, 73.6% and 75.9% in South Africa using NPA and sputum samples, 54.7% in Tanzania [4,6,17-19]. The molecular tests were more sensitive in detecting MTB(C) in respiratory samples than in non-respiratory samples as previously described [17,29] which may be explained by the design of the molecular tests originally aimed to work in respiratory samples. We observed no differences in sensitivity and specificity for sputum, GA, or BAL samples, as previously observed [19].

The specificity was lower in smear-positive samples than in smear-negative samples. It might be an artefact resulting from the pooling of different molecular methods or the low specificity of molecular tests for the DNA target or the use of an imperfect gold standard method. Although culture is the recognised current gold standard for TB diagnosis, it still might be an imperfect comparator, possibly leading to failure to diagnose some cases [17].

Similar performance characteristics of INNO-LiPA Rif. TB and Xpert MTB/RIF used for the diagnosis of TB in adults were reported in Italy and the UK [7,29]; however, these results were primarily evaluating a single rapid method (Xpert MTB/RIF) on a single type of sample. High incidence of TB may explain the higher PPV observed in studies conducted outside Europe (100% for sputum and GA in Vietnam, 81.8% and 86.8% for sputum and GA samples respectively in Zambia, 92.8% and 89.7% in South Africa using NPA and sputum samples) [6,17-19], compared with our study.

Our study showed that NTMs were more frequently isolated from children than MTB(C). *M. bovis* BCG was frequently isolated in Latvia and Lithuania where BCG vaccination is a national policy. Our data showed that *M. abscessus* was the predominant NTM isolated from children while previously was the *M. avium* complex [30].

This study has several limitations. The analysis was based on subnational data, leading to possible selection bias and restriction of the geographical representativeness of the study. A diagnostic bias resulting from more invasive samples being taken when there is a higher level of clinical suspicion may cause the higher positivity rates in non-respiratory samples. Two sites could provide 2011 data only. Small case numbers forced 'pooling' of the NAAT results and our approach ignores the possible heterogeneity of results or differences in ROC curve of various tests. The retrospective laboratory-based approach did not allow the investigators to obtain data on HIV status, clinical/radiological findings and TB treatment. A more comprehensive assessment of the success of diagnosing TB in children in Europe is needed. Ideally, new studies should

be led by key organisations in the field of infectious diseases control and prevention, such as ECDC or WHO Regional Office for Europe, and result in effective recommendations.

As long as available evidence for paediatric TB diagnosis remains limited, however, this study is of public health importance as it reviewed routine laboratory work in a non-clinical trial context, allowing conclusions to be drawn based on real-life scenarios. Moreover, the study sites represent the largest laboratories with reference functions. This work has also demonstrated that data from the ERLN-TB can be used to conduct operational research.

Despite the relative success in diagnosing TB when using non-respiratory or GA samples, the level of laboratory confirmation in children remains low, resulting in treatment initiation based on a set of subjective parameters. TB treatment is long and antituberculosis drugs have potential toxicity; therefore while it is important not to risk failing to diagnose TB, overdiagnosis may result in unnecessary psychological or physical stress for children [26]. With this in mind, a more intensive approach to obtaining paediatric samples, including samples other than sputum (in particular GA and non-respiratory samples), is advisable in order to increase the number of laboratory-confirmed cases and give physicians a much greater degree of confidence when administering antituberculosis treatment. Additionally, a major effort is needed to optimise and evaluate molecular assays for analysis of GA or non-respiratory samples, thus making the diagnosis of paediatric TB more accurate.

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## Conflict of interest

None declared.

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## Authors' contributions

Conceived and designed the study: AS, LF, FD, WH, CDM, YB. Collected data: AS, LF, WH, FD, ER, VKJ, EP, GS, CDM, ERLN-TB members, YB. Analysed the data: AS, LF, WH, FD, ER, VKJ, EP, GS, CDM, YB. Wrote the paper: AS, YB. All authors read and approved the final manuscript.

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# ECDC and WHO/Europe joint report on tuberculosis surveillance and monitoring in Europe

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The European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) Regional Office for Europe have jointly launched the sixth report on surveillance and monitoring of tuberculosis (TB) in Europe [1]. The report indicates that, in spite of notable progress in the past decade, TB is still a public health concern in many countries across Europe. High rates of multidrug-resistant (MDR) TB outside the European Union (EU)/European Economic Area (EEA) are of particular concern. Meanwhile EU/EEA countries themselves have a significant number of TB cases among vulnerable population groups, such as people of foreign origin and prisoners.

In 2012, 68,423 cases of TB were reported in 29 EU/EEA countries, 6% less than in 2011, reflecting a decrease in 19 countries. The EU/EEA notification rate was 13.5 per 100,000 population. Eighty per cent of all notified TB cases were newly diagnosed and 70% of new pulmonary TB cases were culture-confirmed. Twenty-seven per cent of all TB cases were of foreign origin, mostly residing in low-incidence countries. Adults were equally affected by TB, while the notification rate in children under the age of 15 years was 3.6 per 100,000, consistent with a slightly decreasing long-term trend. Males were over-represented in almost all EU/EEA Member States and among adults, with the greatest gender imbalance among those aged 45 to 64 years.

An assessment of progress towards TB elimination for the four epidemiological indicators and eight core indicators defined in the report 'Progressing towards TB elimination: A follow-up to the Framework Action Plan to Fight Tuberculosis in the European Union' [2] showed that none of the core indicators was achieved at EU/EEA level.

Notwithstanding this, since 2001, TB incidence has been falling at an average rate of 5.0% per year, which is the fastest decline in the world.

ECDC and WHO/Europe have coordinated the collection and analysis of TB surveillance data across the countries of the WHO European Region (except Liechtenstein, Monaco and San Marino) since 2008.

## References

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2. European Centre for Disease Prevention and Control (ECDC). Progressing towards TB elimination: A follow-up to the Framework Action Plan to Fight Tuberculosis in the European Union. Stockholm: ECDC; 2010. Available from: [http://ecdc.europa.eu/en/publications/publications/101111\\_spr\\_progressing\\_towards\\_tb\\_elimination.pdf](http://ecdc.europa.eu/en/publications/publications/101111_spr_progressing_towards_tb_elimination.pdf)