

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% ^a
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% ^a
Turkey	0	0	191	249	240	263	222	250	262	1,677	20	1%
Total										16,858	2,055	12%

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

^a 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

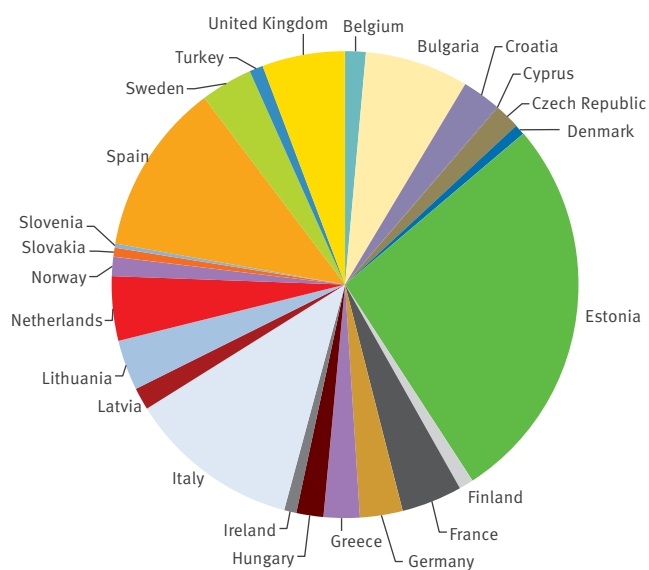
The countries participating in the project reported 16,858 MDR-/XDR-TB cases to the ECDC for the period 2003 to 2011. The total number of MDR-/XDR-TB isolates collected in that period for which VNTR typing data were available amounted to 2,055. Therefore, the coverage of the molecular surveillance for 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

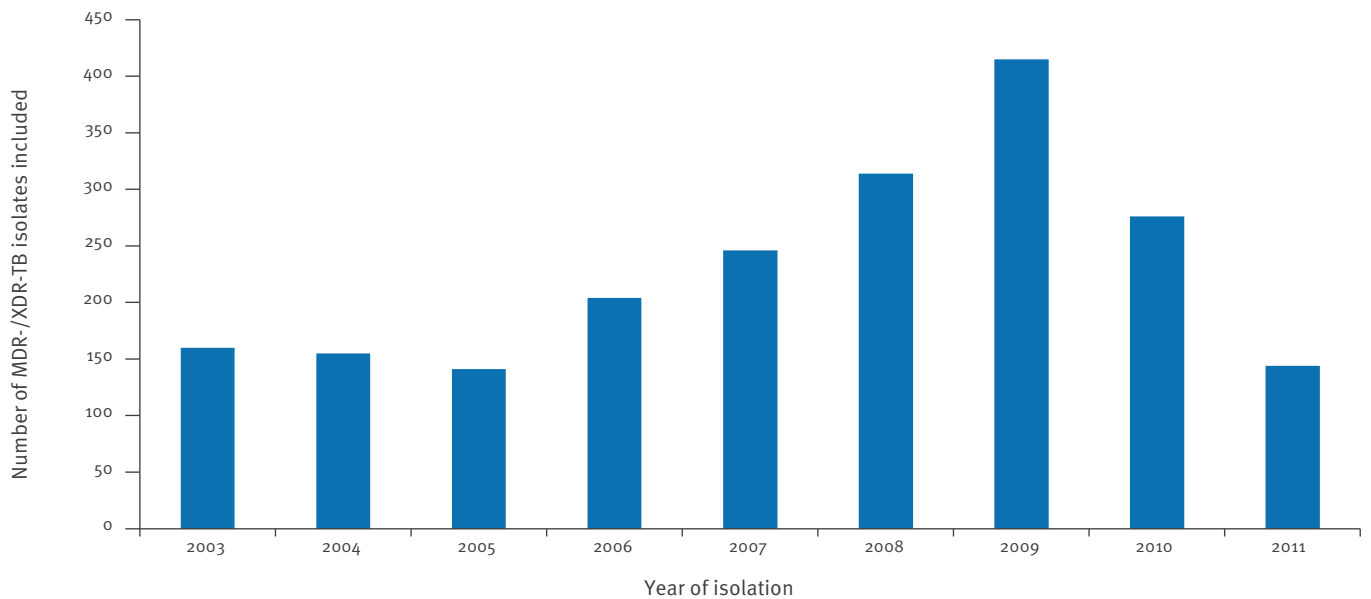
Number of VNTR patterns of multi- and extensively drug-resistant *Mycobacterium tuberculosis* isolates included in the molecular surveillance project, by country of isolation, sampled 2003–2011 (n=2,092)



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.

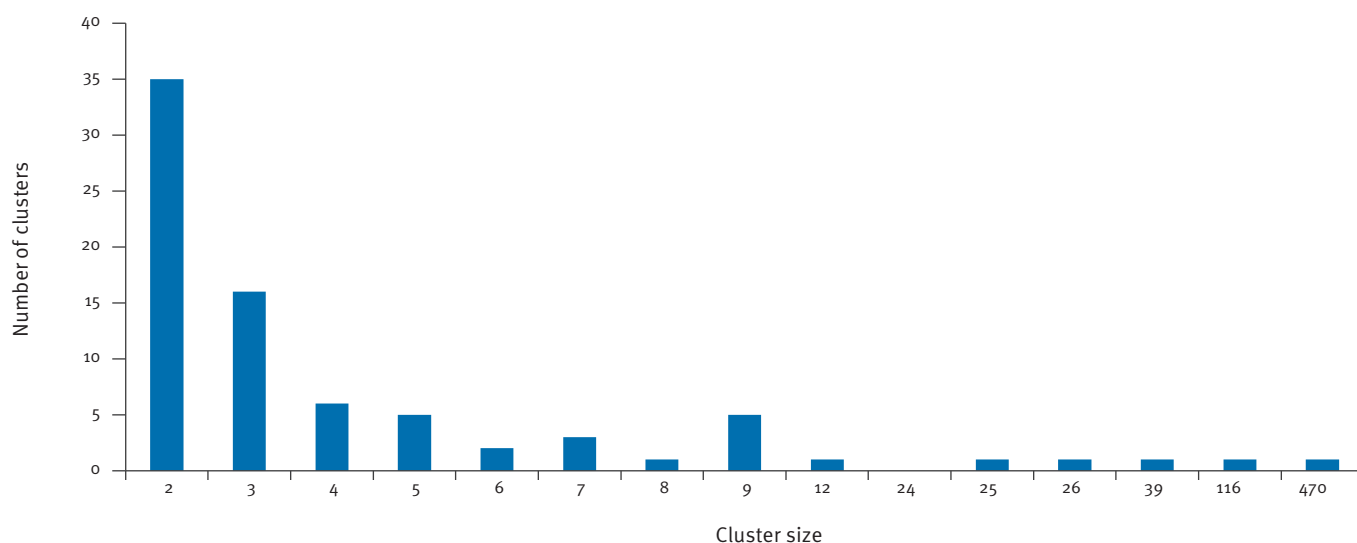
The percentage of samples assigned to a European MDR-/XDR-TB cluster, for the countries which submitted at least 10 isolates to the project database, varied from 0 to 87% by country. Clustering on national level was also analysed in this study and varied from 0 to 92% by country (Figure 4).

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

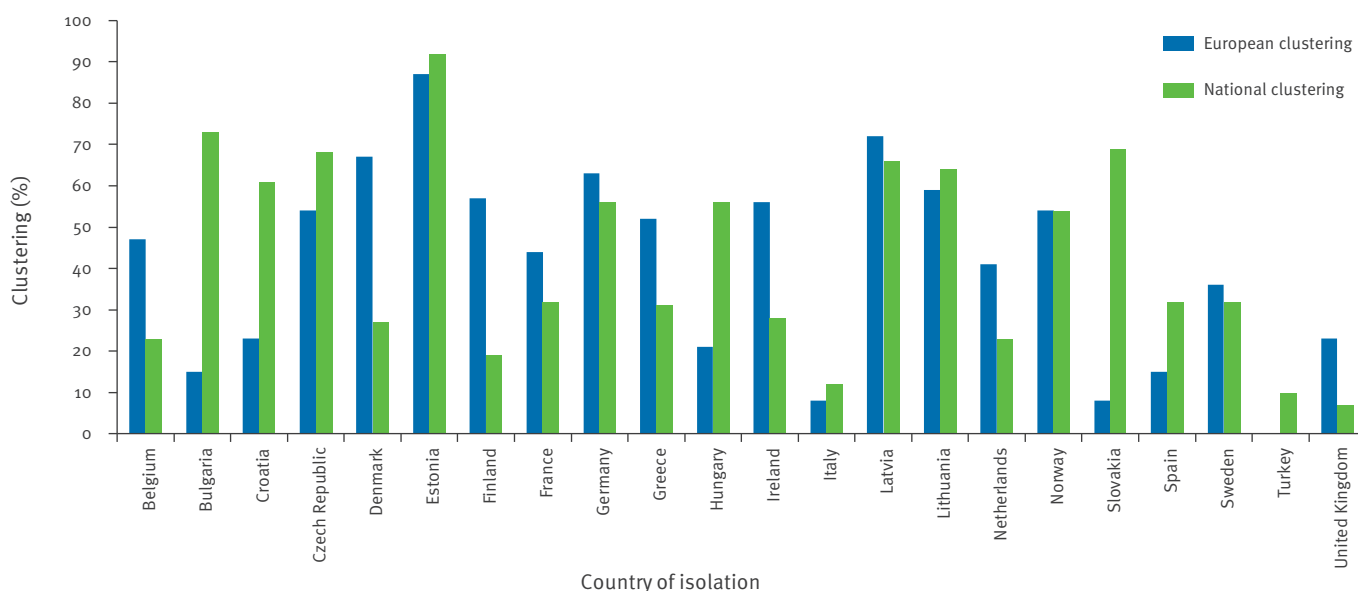


TABLE 2

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.

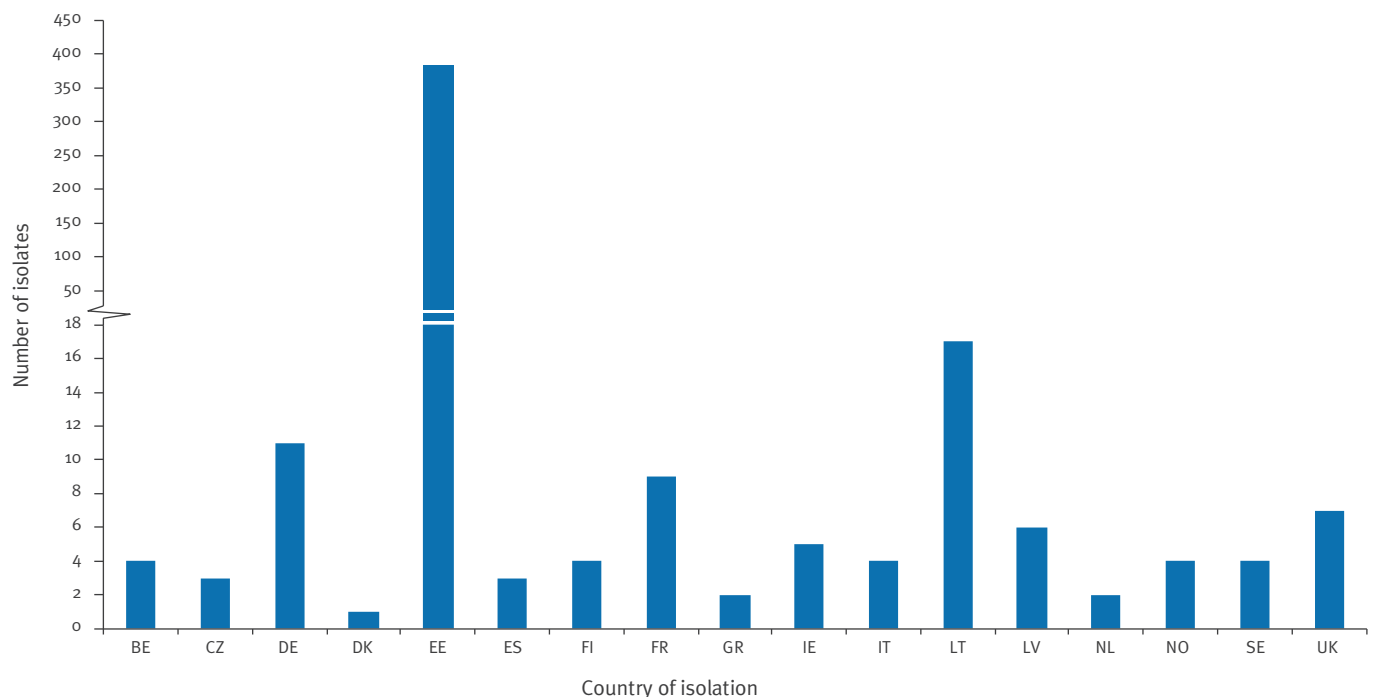
In total, 77% (n=726) of the clustered cases were caused by Beijing genotype strains with 37 different VNTR patterns (the two largest molecular clusters were caused by Beijing genotype strains). Among non-clustered

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

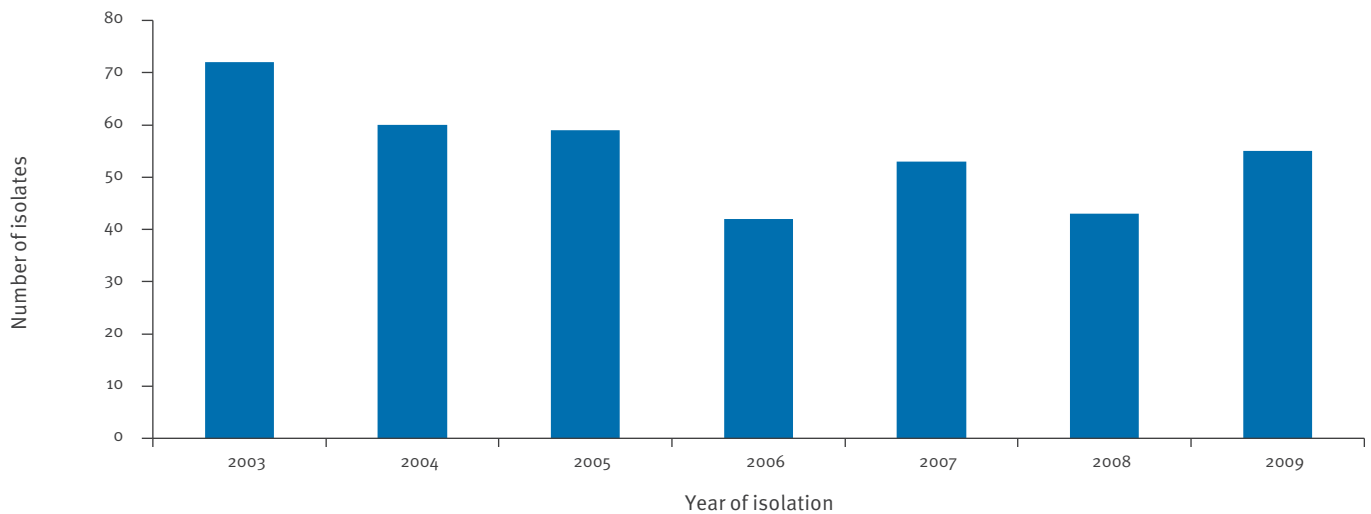
Geographical distribution of cases in the largest European multi- and extensively drug-resistant tuberculosis cluster 2003–2011 (n=470)



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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