Tuberculosis 97 (2016) 147–153

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MOLECULAR ASPECTS

Genomic diversity of *Mycobacterium tuberculosis* Beijing strains isolated in Tuscany, Italy, based on large sequence deletions, SNPs in putative DNA repair genes and MIRU-VNTR polymorphisms

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ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form
15 October 2015
Accepted 20 October 2015

Keywords:
Mycobacterium tuberculosis
Beijing genotype
RD deletions
mutT genes
MIRU-VNTR

SUMMARY

The Beijing genotype of *Mycobacterium tuberculosis* is cause of global concern as it is rapidly spreading worldwide, is considered hypervirulent, and is most often associated to massive spread of MDR/XDR TB, although these epidemiological or pathological properties have not been confirmed for all strains and in all geographic settings. In this paper, to gain new insights into the biogeographical heterogeneity of the Beijing family, we investigated a global sample of Beijing strains (22% from Italian-born, 78% from foreign-born patients) by determining large sequence polymorphism of regions RD105, RD181, RD150 and RD142, single nucleotide polymorphism of putative DNA repair genes *mutT4* and *mutT2* and MIRU-VNTR profiles based on 11 discriminative loci. We found that, although our sample of Beijing strains showed a considerable genomic heterogeneity, yielding both ancient and recent phylogenetic strains, the prevalent successful Beijing subsets were characterized by deletions of RD105 and RD181 and by one nucleotide substitution in one or both *mutT* genes. MIRU-VNTR analysis revealed 47 unique patterns and 9 clusters including a total of 33 isolates (41% of total isolates); the relatively high proportion of Italian-born Beijing TB patients, often occurring in mixed clusters, supports the possibility of an ongoing cross-transmission of the Beijing genotype to autochthonous population. High rates of extra-pulmonary localization and drug-resistance, particularly MDR, frequently reported for Beijing strains in other settings, were not observed in our survey.

1. Introduction

Among the spectrum of genotypes of the *Mycobacterium tuberculosis* complex that prevail worldwide, the Beijing genotype is the one that causes major concern.

Beijing strains are highly endemic throughout much of Eastern and Southeast Asia where are responsible for more than 50% of TB cases, are rapidly spreading worldwide and are most often associated with the massive spread of multidrug-resistant (MDR) tuberculosis (TB). Beijing strains are also considered to be more virulent than other strains [1]; moreover, a number of reports link the Beijing strains with extra-pulmonary TB or with treatment failure and relapse [2–4]. However, the association of Beijing strains with MDR TB and/or with specific pathological or epidemiological properties has not been confirmed for all strains and in all geographic settings [5–8], which suggests the existence of a substantial biogeographical heterogeneity affecting biological properties among the sub-lineages that constitute the Beijing family.

Analysis of genomic polymorphism based on robust markers, such as deletions of large DNA sequences, showed that the Beijing family consists of distinct branches defined by specific deletions designated regions of difference (RD). One large deletion (RD105) was found in all Beijing strains and thus was proposed to serve as a useful marker for the identification of this genotype; additional deletions, namely RD181, RD150 and RD142, further divided the Beijing family into other sub-lineages [9]. Subsequent studies defined the Beijing sub-lineages to a finer scale; in particular, different sequence types, phylogenetically related to the evolutionary pathways suggested by large sequence polymorphisms (LSPs), were detected in a set of Beijing strains of different geographic origins by comparative genomic analysis of single nucleotide polymorphisms (SNPs) in a group of highly polymorphic

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http://dx.doi.org/10.1016/j.tube.2015.10.008
1472-9792/

genes involved in DNA replication, recombination and repair (3R genes) [10]; more recently, in a study of the global spread of the Beijing lineage based on the 24-locus MIRU-VNTR analysis from almost 5000 isolates gathered from a worldwide collection, Beijing strains were classified into 6 major clonal complexes (CC1-CC6) and 1 basal sublineage (BL7) and it was shown that CC1-CC5 included typical/modern strains, whereas CC6 and BL7 included atypical ancestral variants [11].

In this paper we report the genotypic characterization of a collection of Beijing strains isolated in Tuscany, Italy, a region with a low prevalence of TB, but where the ethnic diversity of TB patients, due to the immigration from high-prevalence TB countries, provides an opportunity to study a global sample of Beijing strains [12] and possibly to gain new insights into the biogeographical heterogeneity of the Beijing family. In particular, the purposes of this paper are: (i) to study the genomic polymorphisms based on large sequences deletions and single nucleotide substitutions in *mutT2* and *muT4* putative DNA repair genes; (ii) to determine the molecular profiles of the Beijing isolates by Mycobacterial Interspersed Repetitive Units – Variable Number of Tandem Repeats (MIRU-VNTR) typing for a molecular epidemiology survey of Beijing TB in our setting and to gain insight into genetic relationships between the Beijing isolates.

2. Materials and methods

2.1. Clinical isolates

A total of 80 *M. tuberculosis* strains of Beijing genotype, isolated from 2002 to 2010 from the same number of TB patients resident in Tuscany, Italy, at the Clinical Mycobacteriology Laboratory of the Santa Chiara University Hospital, Pisa, or referred to the laboratory for reference purposes from other community hospitals, were investigated. Drug susceptibility of isolates was determined by the radiometric BACTEC 460 TB or the fluorimetric MGIT 960 systems (Becton Dickinson, Towson, Md., USA) in accordance with the manufacturer's recommendations.

M. tuberculosis isolates were assigned to the Beijing genotype on the basis of deletion of the spacers 1–34 assessed by the standard spoligotyping assay. All the study strains belonged to the genetic group 1, defined on the basis of *katG463* and *gyrA95* alleles polymorphism [13] and had an intact *pks15/1* gene [14].

Genomic DNA for molecular studies was extracted from the bacteria grown on ADC-supplemented Middlebrook 7H9 or Lowenstein-Jensen medium by the cetyltrimethyl-ammonium bromide (CTAB) method.

2.2. RD polymorphism

The identification of genomic deletion RD105, RD181, RD150 and RD142 was performed by PCR using primers previously described [15]. PCR conditions were 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 μM primers, 0.2 mM deoxynucleoside triphosphates, 1 U of Taq polymerase (Dynazyme) and 10 ng DNA per 50 μl of reaction mixture. PCR amplification was performed at the following conditions: 95 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 3 min. Ten-μl aliquots of PCR products were analysed by 2% agarose gel electrophoresis.

2.3. mutT gene polymorphism

Mutations in *mutT* genes were determined either by nucleotide sequencing using oligonucleotide primers pairs designed to amplify a 398 bp fragment of *mutT4* gene and a 675 bp fragment of *mutT2* gene, as previously reported [16], or by a duplex real-time PCR assay developed by ourselves using primers and hybridization probes targeting the *mutT4* and *mutT2* genes by LightCycler instrument (Roche Diagnostics, Germany). Hybridization probes were designed to enable real-time PCR detection of mutations at codon 48 of *mutT4* and at codon 58 of *mutT2* within the same reaction mix [17].

2.4. Mycobacterial interspersed repetitive units – variable number of tandem repeats (MIRU-VNTR) typing

MIRU-VNTR typing was performed by PCR amplification of the following 11 loci selected on the basis of high discriminatory power among the Beijing isolates [18]: MIRU40, MIRU10, 1955, QUB-11b, MIRU26, MIRU31, QUB-26, 1982, 3232, 3820 and 4120. The PCR fragments were analyzed by gel electrophoresis using 2% NuSieve agarose (Cambrex Bio Science Rockland). For each locus, sizes of amplicons were estimated by comparison with 20 bp and 100 bp markers (Superladder-low; GenSura, CA, USA) and the numbers of repetitive units were determined. MIRU-VNTR profile is expressed as a string of 11 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the order given above. The allelic diversity (h) of the MIRU-VNTR loci was calculated using the equation $h = 1 - \sum x_i^2\{n/n-1\}$ where n is the number of isolates and x_i the frequency of the ith allele at the locus [19]. MIRU-VNTR data were analyzed by the MIRU-VNTR*plus* web application available at www.miru-vntrplus.org; MIRU-VNTR profile similarities were visualized by generating a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA); the genetic relationships among the isolates were analyzed by constructing a minimum spanning tree (MST), an undirected network in which all the MIRU-VNTR profiles are linked together with the smallest possible linkages between nearest neighbours, by the UPGMA method.

3. Results and discussion

3.1. Epidemiology

A total of 80 *M. tuberculosis* strains of Beijing genotype isolated in the years 2002–2010 from the same number of TB patients resident in Tuscany, Italy, were studied. As shown in Table 1, Beijing strains were isolated prevalently from male TB patients (male-to-female ratio approximately 2:1) and from people aged 15 to 39 year-old (68%). The country of birth was known for 90% of Beijing patients; 18 (22%) were Italian-born, and 62 (78%) were foreign-born; of these, 54% were from China, 13% from South America (mostly from Peru), 8% from states of the former Soviet Union, 8% from other South Asian countries, 17% from African, European or other unknown countries. Beijing TB had an extra-pulmonary localization in 21% (15/70) of patients, a proportion that is not different from that of non-Beijing strains isolated in the same period in the study area (24%, data not shown), as previously reported [20]. These data contrast with those by Kong et al. [2], who reported that patients infected by the Beijing isolates were nearly three times as likely as patients infected with the non-Beijing isolates to have an extra-thoracic involvement; however, consistent with our observation, a study done in South Africa also failed to demonstrate an association between Beijing strains and the propensity to cause extra-pulmonary disease [21]. In this context, other studies report that extra-thoracic Beijing TB was associated only with sub-lineages bearing specific genomic deletions [22], or that only a recently evolved Beijing sub-lineage showed an increased ability to be transmitted and to cause disease; nonetheless, an association between the sub-lineage and clinical

Table 1
Epidemiological features of Beijing patients and isolates.

Beijing/total isolates (%)	Patients*									Isolates		
	Male/female	Birth		Age group				TB localization		Drug susceptibility†		
		Italian-born	Foreign-born	0–14	15–39	40–64	≥65	Pulmonary	Extra-pulmonary	Isolates tested	Any drug resistance (%)	MDR (%)
80/1325 (6.0%)	51/27	18	62	–	50	16	8	55	15	77	11 (14.3%)	2 (2.6%)

* Gender, country of birth, age and TB localization were unknown for 2, 7, 6 and 10 patients respectively.
† Determined for first-line anti-TB drugs, *i.e.* isoniazid, rifampin, ethambutol and pirazinamide, by the radiometric BACTEC 460 TB or by the fluorimetric MGIT 960 systems (Becton Dickinson, Towson, Md., USA) in accordance with the manufacturer's recommendations. MDR, multi-drug resistance.

parameters could not be demonstrated [15]. In our study, none of the Beijing sub-lineages, defined by RD deletions and *mutT* gene polymorphisms, described ahead in this paper, was found to be preferentially associated with an extra-thoracic involvement. Finally, in our survey, the percentage of Beijing isolates resistant to any first-line anti-TB drug was 14%, not different from that of non-Beijing strains (not shown); only two Beijing isolates were MDR (2.5%).

3.2. Molecular polymorphisms of *M. tuberculosis* Beijing isolates based on RD deletion and mutT mutations

The *M. tuberculosis* Beijing strains were studied to determine the presence or absence of regions RD105, RD181, RD150 and RD142 and the occurrence of mutations in putative DNA repair genes *mutT4* and *mutT2*. As shown in Table 2, no deletion was found in 3 strains that also displayed *mutT4* and *mutT2* tested codons in wild type configuration; the absence of deletion RD105, which is usually considered a marker of the Beijing family [9], is not unprecedented and likely defines very early ancestral Beijing variants in the evolutionary pathway of the Beijing lineage [23]. Ten isolates showed only deletion RD105, all of them with *mutT4* and *mutT2* codons in wild type configuration. A total of 64 isolates, i.e. the prevalent subset, displayed deletions RD105 and RD181; of these, 4 isolates had *mutT4* and *mutT2* genes in wild type configuration; 14 isolates had a *mutT4* gene mutated at codon position 48 and *mutT2* gene in wild type configuration; 46 isolates had both *mutT4* and *mutT2* genes mutated at codon positions 48 and 58, respectively. Finally, 3 isolates showed three RD deletions (RD105, RD181 and RD150) and mutated *mutT4* and *mutT2* genes. Deletion of RD142 was not found in our strain collection.

3.3. MIRU-VNTR analysis of *M. tuberculosis* Beijing isolates

The genetic diversity of the 80 *M. tuberculosis* Beijing strains was further investigated by determining the polymorphism of a set of 11 MIRU-VNTR loci; tested loci included seven conventional MIRU-VNTR loci, that tested sufficiently polymorphic in preliminary assays, and four loci selected on the basis of high discriminatory power among the Beijing isolates according to the recent paper by Allix-Béguec et al. [18]; other conventional MIRU-VNTR loci were excluded from the analysis because of their poor discriminatory power in preliminary assays. We first quantified the resolution provided by each MIRU-VNTR locus by calculating its allelic diversity, which depends upon both the number and the distribution of the alleles, according to Selander et al. [19]; as shown in Table 3, the allelic diversity (h) of the MIRU-VNTR loci of our collection was relatively low only for 3 loci (MIRU40, MIRU10, and MIRU31), but sufficiently high ($h > 0.50$) for the remaining 8 loci, thus confirming the high discriminatory power of these loci for the Beijing isolates [18].

Table 2
Molecular polymorphisms of *M. tuberculosis* isolates of Beijing genotype based on RD regions and *mutT* gene mutations.

No. of isolates	RD region*				Mutations in *mutT* genes†	
	105	181	150	142	*mutT4*	*mutT2*
3	+	+	+	+	wt	wt
10	–	+	+	+	wt	wt
4	–	–	+	+	wt	wt
14	–	–	+	+	mut	wt
46	–	–	+	+	mut	mut
3	–	–	–	+	mut	mut

* + and – indicate presence or absence, respectively, of the specific RD genomic region.
† wt, wild-type codon; mut, mutated codon. Mutations: CGG to GGG (Arg to Gly) at codon position 48 for *mutT4*; GGA to CGA (Gly to Arg) at position 58 for *mutT2*.

The MIRU-VNTR analysis was used to construct a dendrogram, reported in Figure 1, in which the MIRU-VNTR patterns, ordered by similarity, are detailed with additional genomic information (*i.e.*, RD deletions and *mutT* gene mutations), and with epidemiological information (*i.e.*, pulmonary vs extra-pulmonary localization of TB and patients' country of birth). Our MIRU-VNTR analysis revealed 56 distinct MIRU-VNTR patterns; of these, 47 patterns were unique, while 9 patterns were shared by 2 or more isolates, thus yielding 9 clusters including a total of 33 isolates. IS*6100*-RFLP fingerprints, available for most of clustered isolates, confirmed the identity of the MIRU-VNTR-clustered isolates (data not shown). Notably, three MIRU-VNTR clusters, labelled A to C in the figure, also sharing the RD deletion and *mutT* mutation patterns, included relatively high numbers of isolates (*i.e.*, 10, 5, and 6 isolates, respectively); clusters A and C were mixed clusters including both foreign-born (China, prevalently) and Italian-born patients, while cluster B included 5 patients from the same country (Peru). Although it is known that TB patients may occasionally occur in a cluster by chance, through a coincidental reactivation during the observation period, thus generating false clusters including strains without any epidemiological link, the VNTR-MIRU clusters detected in our study appear to be true clusters as they generally include strains isolated in the same geographical area during a one-two years time period, thus establishing place-and-time links indicative of recent TB transmission. As it is assumed that each TB cluster includes one source case, whereas the other clustered cases are considered the result of recent TB transmission, it possible to calculated the active transmission rate in the study area as (33-9)/80 (n–1 method), that yields a value as high as 30.0%. This rate of transmission appears to be significantly higher than that of non-Beijing strains (14.9%) previously reported in the study region [12], and is similar to that found by Moro et al. [24] in the area of Milan, where 28.1% of total TB cases in a population with a higher percentage of AIDS patients were attributed to recent transmission. The high transmission rate of the Beijing strains also in our settings confirms the high transmissibility of this

Table 3
Determination of allelic diversity at each MIRU-VNTR locus of 80 isolates of the Beijing family.

TR copies*	Number of isolates at MIRU-VNTR locus										
	MIRU40	MIRU10	1955	Qub-11b	MIRU26	MIRU31	Qub-26	1982	3232	3820	4120
0	2	1	1					1			1
1	3	4	7				2			1	
2	70	71	2	6	2				5		1
3	5	3	23	2	2	4	1	1		2	2
4		1	44	6	12	7			1		2
5			3	15	11	62	2	1		4	3
6				45	11	6	1	2	2		3
7				6	39		16	10	2		3
8					1	1	52	46		1	12
9							4		11	1	11
10					2		2	17	5	4	37
11								1	6	5	2
12								1	3	15	2
13									25	1	
14									11	19	
15									5	10	
16									1	13	
18									1	1	
19									1	1	
20											
22									1		1
24										2	
Allelic diversity	0.22	0.20	0.61	0.63	0.70	0.39	0.53	0.60	0.85	0.85	0.73

The allelic diversity (h) was calculated using the equation $h = 1 - \sum x_i^2\{n/n-1\}$, where n is the number of isolates and x_i the frequency of the ith allele at the MIRU-VNTR locus [19].

* Number of tandem repeats (TR) copies at the MIRU-VNTR locus.

genotype [25–27]. In this regard, it is worth underscoring that, contrarily to other *M. tuberculosis* strain families endemic in remote geographic areas, such as the spoligotype-defined families East-African-Indian (EAI), Central Asian (CAS) and Africanum, which are found almost exclusively in foreign-born patients in our setting [20], 22.5% (18/80) of Beijing TB cases in our survey occurred in Italian-born patients, thus indicating an ongoing cross-transmission of the Beijing genotype to autochthonous population.

3.4. Phylogenetic relationships between M. tuberculosis Beijing isolates

To visualize the genetic relationships between the study isolates, we constructed a minimum-spanning tree (MST) on the basis of the MIRU-VNTR profiles (Figure 2). In our analysis, the phylogenetically most "ancient" strains, *i.e.* the ones showing wild-type *mutT* genes [28], are scattered at the periphery of the MST; these strains include all the strains with no RD deletion, i.e. the RD105+ early ancestral Beijing variants mentioned in Section 3.2, or with the RD105 deletion alone, and part of strains with both RD105 and RD181 deletions. Strains with mutated *mutT4* and wild-type *mutT2*, which include exclusively RD105-and RD181-deleted strains, form two star-like clonal complexes typical of expanding populations, while the RD105/RD181-deleted, *mutT4*/*mutT2* mutated strains form a large clonal complex of strains linked together, representing the prevalent expanding strain population in our setting.

Other studies have reported Beijing sub-lineages bearing the RD and *mutT* polymorphisms (i.e., RD105/RD181-deletions and *mutT4*/*mutT2* SNPs) detected in the prevalent sub-lineage of our setting; in particular, Mestre et al. [10] described, in different part of the world, a phylogenetically recent prevalent sequence type, named Bmyc10, characterized by the RD181 deletion and SNPs in the putative DNA repair genes *mutT2*, *muT4* and *ogt*; more recently, Merker et al. [11] reported that CC4, one of the six major Beijing clonal complexes, typical of southern and eastern Asia, bears the same RD and *mutT* polymorphisms as our prevalent sub-lineage. In this regard, however, it has to point out that in the Merker's paper [11] the CC2 complex, typical of Europe, Russia and Central Asia, is reported as largely predominant in Italy; the discrepancy with our findings might be possibly attributed the demographic characteristic of the foreign-born population in our region that includes a large Chinese community.

4. Conclusions

The molecular-epidemiological analysis of TB caused by *M. tuberculosis* Beijing strains in our setting confirms the high transmissibility of this family of strains, as shown by the high percentage of clustered isolates. Moreover, the relatively high proportion of Italian-born Beijing TB patients, often occurring in mixed clusters, supports the possibility of significant cross-transmission between foreign-born and autochthonous individuals, regardless of the fact that this event is generally considered epidemiologically irrelevant in low-incidence western countries receiving immigrants [29]. Apart from this reason of concern, high rates of extra-pulmonary localization and MDR occurrence, frequently reported for Beijing strains in other settings, were not observed in our survey; this confirms the existence of a substantial biogeographical heterogeneity affecting biological properties among the members that constitute the Beijing family. In this regard it is worthy to note that the highly prevalent Beijing sub-lineages detected in our study show, aside from deletions of RD105 and RD181, a nucleotide substitution in one or both putative DNA repair *mutT* genes, a polymorphism that is characteristic and unique of the Beijing lineage [28]; as hypermutation is considered a factor conferring selective advantages [30], it is tempting to speculate that Beijing strains

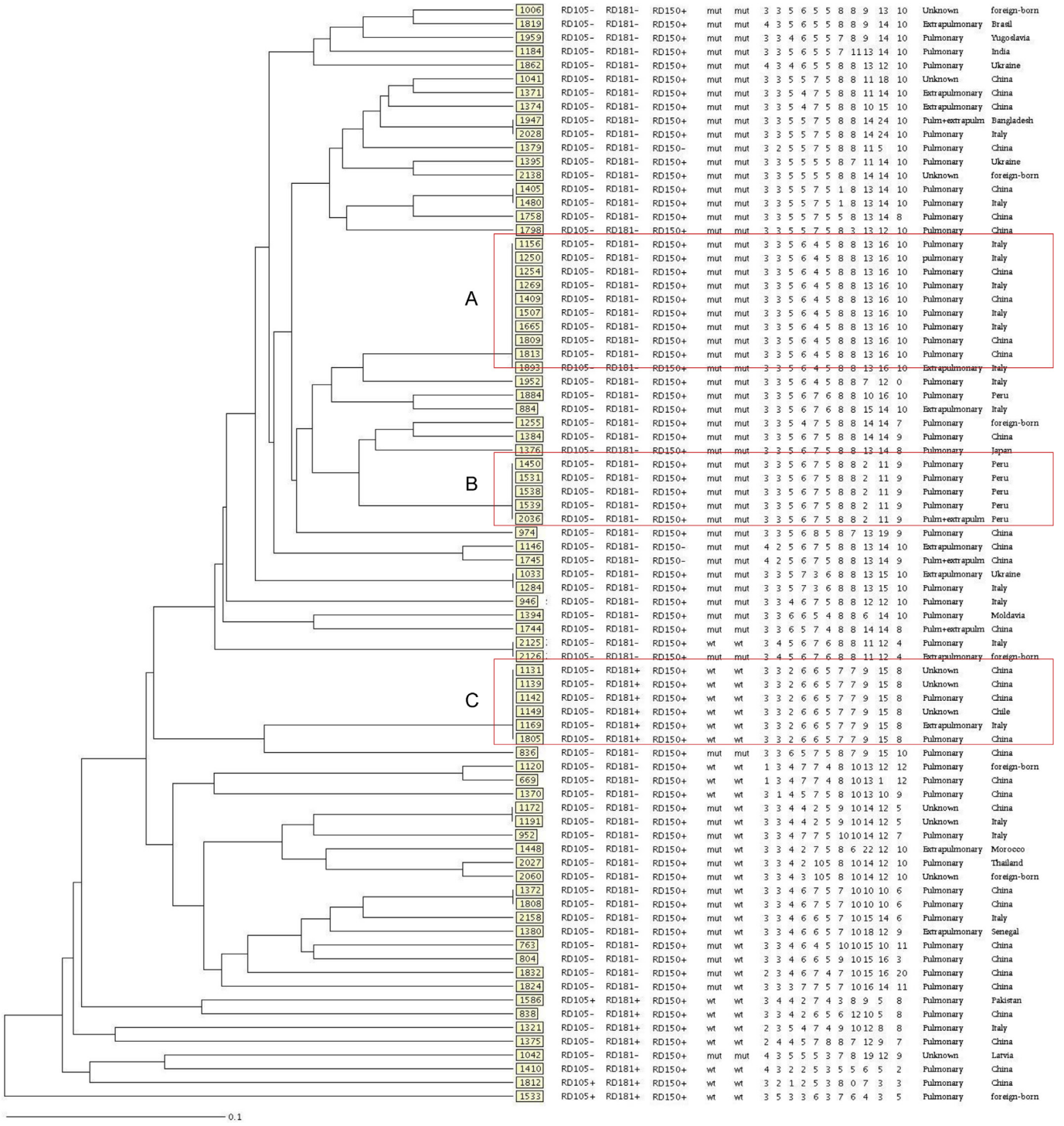

Figure 1. Dendrogram constructed on the basis of MIRU-VNTR profiles of a set of 11 loci (see text) of 80 *M. tuberculosis* Beijing isolates; each profile is associated with data on RD deletions, SNPs in *mutT* genes, site of TB infection and patient's country of birth. The dendrogram was generated using the UPGMA method by the MIRU-VNTR*plus* web application available at www.miru-vntrplus.org. The columns 1 to 19 on the right of the dendrogram represent respectively: 1) isolate ID code (boxed); 2–4) RD105, RD181 and RD150 deletions; 5–6) wild-type (wt) or mutated (mut) codon configuration of *mutT4* and *mutT2* genes; 7–17) isolate MIRU-VNTR profiles expressed as a string of 11 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the order stated in the paper; 18) pulmonary/extrapulmorary localization of TB; 19) patient's country of birth. The largest clusters, labelled A, B and C, are included in boxes.

might benefit from such molecular feature through increased transmissibility or resistance to harmful conditions, such as the exposure to anti-mycobacterial drugs. Although the missense alterations detected in our survey in *mutT* genes do not appear to increase drug resistance rate of the Beijing isolates, which are mostly primary isolates, it cannot be ruled out that drug resistance may arise when strains are exposed to the selective pressure of anti-TB therapy. On this subject, further studies directly investigating the response of Beijing strains to anti-TB drugs are necessary.

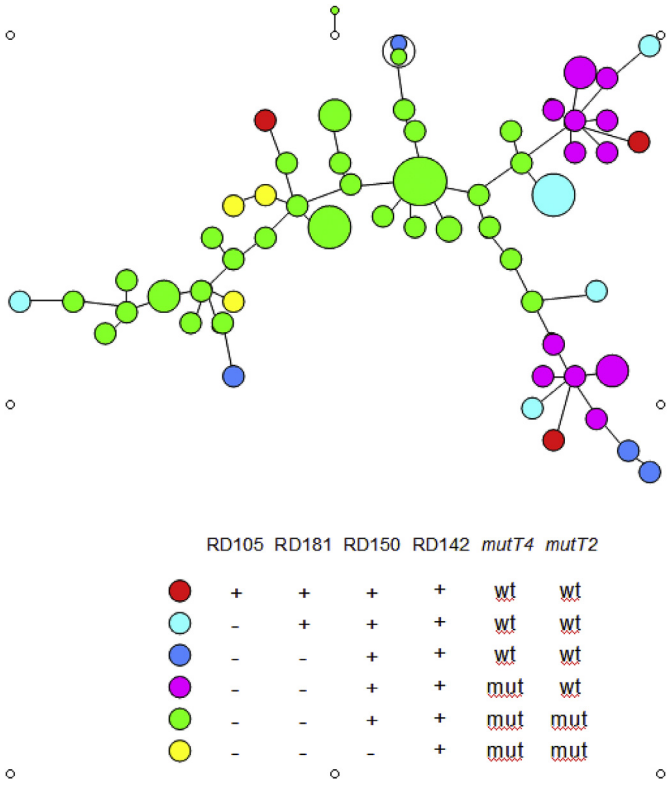

Figure 2. Minimum spanning tree based on MIRU-VNTR profiles of a set of 11 loci (see text) of *M. tuberculosis* Beijing isolates bearing deletions RD105, RD181, RD150 and RD142 and wild-type (wt) or mutated (mut) codons of *mutT4* and *mutT2* genes (the different combinations are indicated in different colours in the legend at the bottom of the figure). Each small-size circle represents a single isolate; larger circles represent clusters of 2–10 isolates, depending on the circle size, with identical MIRU-VNTR profiles; one circle includes 2 isolates with identical MIRU-VNTR profiles but differing for RD deletions. The length of the lines connecting isolates or clusters are not proportional to the number of allelic variation between the isolates. The tree was generated using the UPGMA method by the MIRU-VNTRplus web application available at www.miru-vntrplus.org.

Funding: This work was financially supported by the University of Pisa, Grant "Fondi di Ateneo, 2013–2014".

Competing interests: None declared.

Ethical approval: Not required.

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