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Genetic diversity of human isolates of *Mycobacterium bovis* assessed by spoligotyping and Variable Number Tandem Repeat genotyping

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ABSTRACT

A collection of clinical isolates including 9 *Mycobacterium bovis* bacille Calmette–Guérin (BCG), 37 *M. bovis* and 1 isolate identified as *M. bovis*/*caprae* intermediate, recovered from humans in Tuscany, Italy, from 1990 to 2009, was genotyped by spoligotyping and Variable Number Tandem Repeat (VNTR) typing. Spoligotyping detected 15 unique profiles; the "BCG-like" SIT482/SB0120 spoligotype was largely prevalent accounting for 63.8% of isolates. VNTR typing, based on the 15 VNTR loci commonly tested for *Mycobacterium tuberculosis*, detected 29 unique profiles; only 8 VNTR loci (VNTR 43, MIRU 04, QUB-11b, ETR-A, VNTR 47, MIRU 31, QUB-26 and VNTR 53) provided a satisfactory allelic diversity in the VNTR analysis. Combined together, spoligotyping and VNTR typing yielded 33 unique patterns and 5 clusters including a total of 19 isolates. Clustered isolates, further typed for additional 9 VNTR loci, finally yielded 3 distinct clusters including 3 *M. bovis* BCG isolates each, and 1 cluster of 6 *M. bovis* isolates. Minimum spanning tree analysis showed that, in spite of the many distinct VNTR profiles, most *M. bovis* isolates displayed a high phylogenetic proximity, due to the variation of a single VNTR allele, thus indicating that the population of human *M. bovis* isolates in our setting is relatively homogeneous and conserved.

1. Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTBC), is the major causative agent of bovine tuberculosis (TB). In contrast to *M. tuberculosis*, that is largely host restricted to humans, *M. bovis* infects a wide range of domestic and wild animals, mainly cattle and other bovids, and can also be recovered from other mammals including humans, where it causes a disease indistinguishable from that caused by *M. tuberculosis*.

In many industrialized countries, *M. bovis* has been eradicated from cattle or reduced to very low levels and the bovine TB has essentially been reduced to a disease of economic importance, but in low-income developing countries *M. bovis* represents a public health concern affecting livestock, humans and ecosystem (Michel et al., 2010). In Italy, as a result of a national eradication program of bovine TB, the official TB-free status was achieved starting from 1970 in some Italian areas, but in the rest of the national territory bovine TB still persists with different prevalence rates (Boniotti et al., 2009) and *M. bovis* isolates are reported in human TB cases. Actually, in our region, Tuscany (Italy), in spite of the recent achievement of the bovine TB-free status, 1.7% of over one thousand human TB cases that occurred during years 2002–2005 was due to *M. bovis* (Lari et al., 2009), thus indicating that *M. bovis* TB still remains a potential hazard in Italy.

M. bovis is transmitted within and between domestic and wildlife animals, as well as from animals to humans or vice versa. Contaminated food (especially milk) or direct contact with infected animals is considered to be the primary route by which *M. bovis* infects humans (O'Reilly and Daborn, 1995). Direct human-to-human transmission seems to be rare (O'Reilly and Daborn, 1995; Blazquez et al., 1997; Evans et al., 2007) and in most circumstances the origin of *M. bovis* infection for humans remains undetermined despite its importance.

In the last years, molecular typing of isolates has become a valuable tool in the study of *M. bovis* epidemiology, as well as to determine the phylogenetic relationships among the strains. The most common epidemiological molecular typing method applied to *M. bovis* strains is spoligotyping, a method that identifies the polymorphism based on the presence or absence (deletion) of single or multiple spacer sequences interspersed in the direct repeat (DR) region of chromosome (Kamerbeek et al., 1997). However, because the loss of spoligotype spacer sequences is relatively frequent among the MTBC members, identical spoligotype patterns can occur independently in genetically unrelated

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lineages, and therefore the deletion of spoligotype spacers may be an unreliable indicator of epidemiological and/or phylogenetic relationships (Smith et al., 2006; Warren et al., 2002). For this reason, recently molecular analysis based on the polymorphism of chromosomal loci containing variable number of tandem repeats (VNTRs) – often combined with spoligotyping – has emerged as an appropriate typing method for the highest levels of epidemiologic discrimination for *M. bovis* strains. In the conventional VNTR method proposed by Supply et al. (2006) for the analysis of MTBC strains, sets of 12, 15 or 24 VNTR loci, including the so-called Exact Tandem Repeats (ETR) (Frothingham and Meeker-O'Connell, 1998), the Mycobacterial Interspersed Repetitive Units (MIRUs) (Supply et al., 2000; Mazars et al., 2001) and sets of Queen's University Belfast (QUB) VNTRs (Roring et al., 2004; Skuce et al., 2002), are analyzed providing different degrees of discrimination among isolates. More recently, additional VNTR loci have been proposed for the optimal discrimination among MTBC isolates (Velji et al., 2009), including *M. bovis* isolates of animal origin (Hilty et al., 2005; Allix et al., 2006).

In the present paper we have determined the polymorphism of the 15 VNTR loci commonly tested for *M. tuberculosis* human isolates to assess the usefulness of the VNTR analysis, in combination with spoligotyping, for the highest discrimination among *M. bovis* human isolates, to investigate their genotypic diversity and to contribute to the knowledge of *M. bovis* epidemiology in humans in Italy.

2. Materials and methods

2.1. Clinical isolates

A collection of 47 *M. bovis* clinical isolates, recovered from 38 Italian-born and 9 foreign-born patients from 1990 to 2009 in Tuscany, Italy, were studied. The *M. bovis* isolates were identified by the use of DNA molecular probes specific for the *M. tuberculosis* complex (Accuprobe, Gen-Probe) and by the commercial Genotype MTBC assay (Hain Lifescience GmbH, Nehren, Germany). This assay targets fragments of the *rpoB* gene, the RD1 region of *M. tuberculosis* and *M. bovis*, which is absent from *M. bovis* bacille Calmette–Guérin (BCG), and the *gyrB* polymorphism, characterized by mutations suitable to differentiate most of the species belonging to the *M. tuberculosis* complex. All the isolates

Table 1
Spoligotyping and VNTR analysis of 47 *M. bovis* clinical isolates from humans.

No of isolates	Identification[a]	Spoligotyping[b]			VNTR[c]
		Designation by		Binary pattern	
		SpolDB4	Mbovis.org		
3	*M. bovis* BCG	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	062'223135253250
3	*M. bovis* BCG	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	052'223135253250
3	*M. bovis* BCG	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053'223135253250
6	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223144453241
4	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453241
1	*M. bovis*	SIT820	SB0856	■■□■■■■■□■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453241
1	*M. bovis*	SIT0	SB0927	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453241
1	*M. bovis*	SIT1932	SB0950	■■□■■■■■□■■■□■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223125453251
1	*M. bovis*	SIT820	SB0856	■■□■■■■■□■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223125453251
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	033223144453241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	033223145453241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223134453251
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223134455241
1	*M. bovis/caprae*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145454241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223135453251
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145253241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145452241
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453221
1	*M. bovis*	SIT482	SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	253223325453251
1	*M. bovis*	SIT665	SB0134	■■□□□■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145444251
1	*M. bovis*	SIT672	SB0269	■■□■■□■□□□□□■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	253222327453251
1	*M. bovis*	SIT977	SB0867	□□□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223134453221
1	*M. bovis*	SIT977	SB0867	□□□■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	052222145442221
1	*M. bovis*	SIT1842	SB1060	■□□■■■■■■□■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223135453241
1	*M. bovis*	SIT1932	SB0950	■■□■■■■■□■■■□■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223144452241
1	*M. bovis*	SIT0	SB1205	■■□■■■■■□■■■□□□□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223145453251
1	*M. bovis*	SIT0	SB1209	■■□■■■■□□■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	043324145453241
1	*M. bovis*	SIT0	SB1208	■■□■■□■■□■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	233223325453241
1	*M. bovis*	SIT0	SB1206	■■□■■□□■□■■■■■■□■■■■■■■■■■□□■■■■■■■■■□□□□□	043223144453241
1	*M. bovis*	SIT0	SB1207	■■□■■□□■□■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□□	073223144453221
1	*M. bovis*	SIT0	SB1383	■■□■■■■□■□■■■□■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	053223142453251
1	*M. bovis*	SIT0	SB0934	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■□■■□□□□□	053223145443241

[a]According to RD1 region and *gyrB* polymorphisms assayed by the Genotype MTBC test (see Section 2 for detail).
[b]Spoligotype designation was assigned according to the definition in SpolDB4 and in the Mbovis.org databases; the binary pattern includes symbols "■" and "□" that indicate presence or absence of the specific spacer at positions 1–43 in the DR locus.
[c]VNTR profile is expressed as a string of 15 numbers, each representing the number of tandem repeats at a given VNTR position in the following order: VNTR 42, VNTR 43, MIRU 04, MIRU 40, MIRU 10, MIRU 16, 1955, QUB-11b, ETR-A, VNTR 47, MIRU 26, MIRU 31, VNTR 52, QUB-26, and VNTR 53. For *M. bovis* BCG isolates, the profile of the 3rd VNTR locus assayed (MIRU 04) is given with the symbol ′, indicating an allelic variant lacking in a 53 base-pair segment at the 3′ end. Identical VNTR profiles of isolates showing distinct spoligotype patterns are boxed.

hybridized with the *rpoB* probe, thus confirming their belonging to the *M. tuberculosis* complex. Forty-six strains had *gyrB* mutations typical of *M. bovis* and BCG; nine of these strains lacked RD1 and were identified as *M. bovis* BCG. One isolate presenting RD1 had the *gyrB* mutations typical of *Mycobacterium caprae*, but the spoligotype profile typical of *M. bovis* (see Table 1); accordingly, this isolate was identified as *M. bovis*/*caprae* intermediate.

2.2. Spoligotyping

Spoligotyping analysis of strains was performed basically as described by Kamerbeek et al. (1997). The spoligotypes of 42 isolates were known before the analysis (Lari et al., 2006); five recent isolates were newly spoligotyped. Spoligotypes in binary format were compared with the SITVIT2 database (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html), an updated version of the previously released SpolDB4 database (Brudey et al., 2006) containing at the time of the present study genotyping information on about 70,000 MTBC clinical isolates from 160 countries, and with the *M. bovis*-specialized Mbovis.org database (http://www.mbovis.org). Both spoligotype designations are reported throughout the paper.

2.3. Variable Number Tandem Repeat (VNTR) typing

VNTR typing was performed by PCR amplification of the following 15 loci, as described by Supply et al. (2006): VNTR 42, VNTR 43, MIRU 04, MIRU 40, MIRU 10, MIRU 16, 1955, QUB-11b, ETR-A, VNTR 47, MIRU 26, MIRU 31, VNTR 52, QUB-26, and VNTR 53. 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. VNTR profile is expressed as a string of 15 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the order given above.

Isolates occurring in clusters, i.e., sharing identical VNTR and spoligotype profiles, were further analyzed for VNTR loci MIRU 42, MIRU 20, VNTR 46, VNTR 48, MIRU 23, MIRU 24, MIRU 27, VNTR 49, and MIRU 39, according to Supply et al. (2006).

2.4. Genetic relationships analysis

The genetic relationships between the VNTR patterns in our isolate collection were investigated by drawing a minimum spanning tree by the MIRU-VNTR*plus* web application available at www.miru-vntrplus.org. The minimum spanning tree is an undirected network in which all the samples within the population studied are linked together with the smallest possible linkages between nearest neighbours.

3. Results and discussion

3.1. Spoligotyping and VNTR typing

A total of 47 mycobacterial clinical isolates from humans, including 9 *M. bovis* BCG isolates, 37 *M. bovis* isolates and 1 isolate identified as *M. bovis*/*caprae* intermediate, were genotyped by spoligotyping and VNTR typing. Data are reported in Table 1. Spoligotyping detected 15 unique profiles among isolates. The prevalent spoligotype was the SIT482/SB120 (usually referred to as "BCG-like"), detected in 30 isolates (63.8%); this spoligotype was found in all the *M. bovis* BCG isolates, as well as in the reference control strains *M. bovis* BCG Pasteur and *M. bovis* BCG Tice (data not shown). Spoligotyping also detected 3 spoligotypes shared by 2 human isolates (SIT820/SB0586, SIT977/SB0867 and SIT1932/SB0950), and 11 unique spoligotypes; 3 of these were reported in both reference databases (SIT665/SB0134, SIT672/SB0269 and SIT1842/SB1060) and 8 were reported only in the Mbovis.org database (i.e., SB0927, SB1205, SB1209, SB1208, SB1206, SB1207, SB1383, SB0934); according to the rules of the SITVIT2-SpolDB4 database, these isolates were designed as SIT0 in Table 1.

VNTR analysis was more discriminatory as 29 unique VNTR profiles were detected. Of these, 6 profiles were shared by 2 or more isolates, thus yielding 6 clusters including a total of 24 isolates. Three clusters of 3 isolates each included the *M. bovis* BCG isolates; notably, all the BCG isolates showed an allelic variant of the 3rd VNTR locus assayed (i.e., MIRU 04) lacking in a 53 base-pair segment at the 3′-terminus of the locus, as previously reported (Magdalena et al., 1998; Lari et al., 2006). The other VNTR clusters included 6, 7, and 2 *M. bovis* isolates, respectively.

Spoligotyping and VNTR analysis, combined together, yielded the highest discrimination among the isolates, as 2 VNTR-defined clusters (VNTR 053223145453241 and VNTR 053223125453251), boxed in Table 1, including respectively 7 and 2 isolates, were split due to differences in the spoligotype profiles of the isolates. In summary, the molecular analysis based on the two typing techniques combined together yielded 33 unique patterns and 5 clusters including a total of 19 isolates, i.e., 3 clusters of *M. bovis* BCG isolates, a fourth cluster including 6 SIT482/SB0120 *M. bovis* isolates sharing VNTR 053223144453241, and a fifth cluster including 4 SIT482/SB0120 isolates sharing VNTR 053223145453241.

All clustered isolates were further analyzed for additional 9 VNTR loci (i.e., MIRU 42, MIRU 20, VNTR 46, VNTR 48, MIRU 23, MIRU 24, MIRU 27, VNTR 49, MIRU 39) to complete the 24-loci set proposed by Supply et al. (2006), but no further discrimination was achieved, with the exception of the 4 VNTR 053223145453241-SIT482/SB0120 isolates that showed 4 distinct allelic variations at locus VNTR 48, alias ETR-B (respectively 2, 3, 4 and 5 tandem repeats; data not shown). Thus, apart the 3 distinct BCG clusters, only 1 cluster of 6 identical *M. bovis* isolates was finally detected in our strain collection.

3.2. VNTR allelic diversity of M. bovis human isolates

In the VNTR typing assay of *M. bovis* human isolates based on the set of the 15 loci commonly tested for *M. tuberculosis* human isolates (Supply et al., 2006), certain loci were poorly discriminatory as they displayed only a single allele (MIRU 10 and VNTR 52) or alleles that were much more frequent than others (VNTR 42, MIRU 40, MIRU 16, 1955 and MIRU 26); only 8 loci (VNTR 43, MIRU 04, QUB-11b, ETR-A, VNTR 47, MIRU 31, QUB-26 and VNTR 53) finally provided a satisfactory discriminatory power. The resolution provided by each locus in the VNTR typing assay was quantified by calculating its allelic diversity, depending upon both the number and the distribution of the alleles. As shown in Table 2, the allelic diversity (h) of the VNTR loci, 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 (Selander et al., 1986), was null or extremely low ($h \leq 0.02$) for loci MIRU 40, MIRU 10 and VNTR 52, poorly discriminatory ($h = 0.10$) for loci VNTR 42, MIRU 16, 1955 and MIRU 26, and satisfactory ($0.22 \leq h \leq 0.55$) for loci VNTR 43, MIRU 04, QUB-11b, ETR-A, VNTR 47, MIRU 31, QUB-26 and VNTR 53.

Ten of the 15 VNTR loci tested in our study are in common with a 24-loci set assayed by Boniotti et al. (2009) to characterize a collection of SIT482/SB0120 *M. bovis* animal isolates from Italy. Of these, only one locus (ETR-A) confirms the high allelic diversity found in our human isolates, while loci QUB-26 and QUB-11b, highly discriminative for our human isolates, are less discriminative for

Table 2
Determination of heterogeneity at each VNTR locus of *M. bovis* clinical isolates.

TR copies[a]	Number of isolates at VNTR locus														
	VNTR 42	VNTR 43	MIRU 04	MIRU 40	MIRU 10	MIRU 16	1955	QUB-11b	ETR-A	VNTR 47	MIRU 26	MIRU 31	VNTR 52	QUB-26	VNTR 53
0	44														9
1							44								38
2	3		7	46	47	2		5	1	10		3	47	4	
3		3	40	1		44	3	14				41			
4		2				1		28	13	37	3	2		25	
5		38							32		44	1		18	
6		3													
7		1							1						
8															
Allelic diversity[b]	0.10	0.32	0.24	0.02	0.00	0.10	0.10	0.54	0.45	0.32	0.10	0.22	0.00	0.55	0.29

[a] Number of tandem repeats (TR) copies.
[b] 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 locus.

animal isolates (h = 0.34 and 0.32, respectively), and locus MIRU 04, that shows a relatively high allelic diversity for human isolates (h = 0.24), is absolutely not discriminative for animal isolates (h = 0).

3.3. Genetic relationships among *M. bovis* human isolates

To visualize the genetic relationships between the study isolates, a minimum spanning tree (MST) was constructed by the MIRU-VNTR*plus* web application. The MST, illustrated in Fig. 1, is based on variations from one allele to another due to the loss or gain of one tandem repeat sequence at a single VNTR locus. By this analysis, the 29 VNTR profiles described above yielded two clonal complexes, termed CC1 and CC2, including 18 and 3 unique profiles, respectively. CC1 (light grey in Fig. 1) included a total of 30 isolates, 15 of which clustered in the 3 clusters of 6, 7, and 2 isolates, respectively. Of these, only the first one included isolates sharing identical 24 loci-VNTR profiles and spoligotype, while the other clusters included isolates that differ either in spoligotypes or in locus VNTR 48 (ETR-B), as described above. CC2 (dark grey in Fig. 1), that differed from nearest CC1 isolate for two allelic variations, included exclusively the BCG isolates that were grouped in 3 clusters of 3 isolates each, thus confirming a different selective pressure for these isolates.

Finally, it is to note that, in spite of the many distinct profiles, most *M. bovis* isolates displayed a high phylogenetic proximity, as the genetic difference of one isolate with the nearest neighbour isolate was generally due to the variance of a single VNTR locus. This indicates that the *M. bovis* genotype is relatively homogeneous and conserved, as already reported for other MTBC families such as Beijing and CAS (Velji et al., 2009).

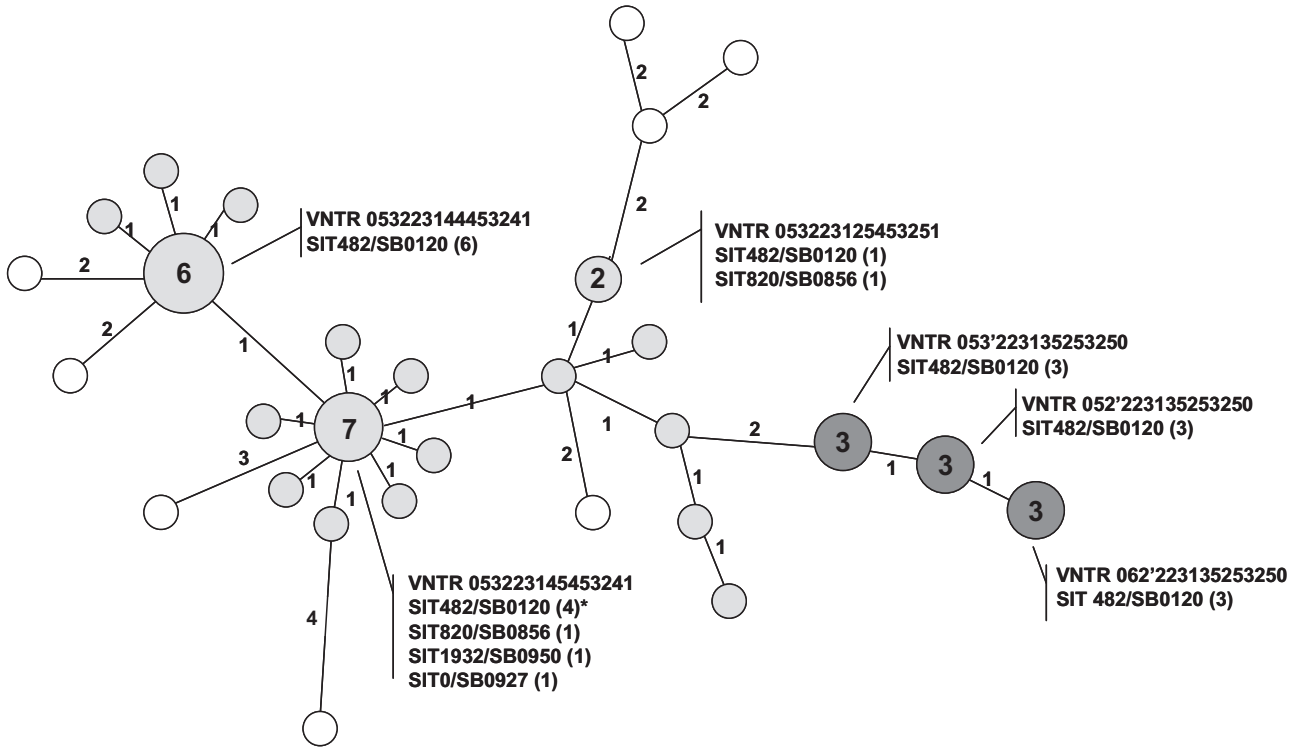

Fig. 1. Minimum spanning tree based on VNTR profiles of a set of 15 loci of 47 *M. bovis* clinical isolates. Numbers inside the circles indicate the number of isolates sharing a given VNTR profile (clusters); for each cluster the VNTR profile, the spoligotype(s) and the number of the isolates (in parenthesis) are given in the callouts; circles without numbers indicate a single VNTR profile. Numbers next to the branches indicate the level of changes induced by loss or gain of VNTR copies at a given locus, yielding a change from one allele to another. Light and dark grey circles indicate VNTR profiles belonging to clonal complexes CC1 and CC2, respectively, detected by the analysis at single locus variance. Asterisk (*) indicates isolates with distinct allelic variations at locus VNTR 48 (ETR-B), not included in the set of 15 loci studied (see text for details).

Table 3
Frequency of VNTR alleles in human and animal SIT482/SB0120 isolates in Italy.

	% of isolates at VNTR locus																			
TR copies[a]	VNTR 43		MIRU 04		MIRU 40		MIRU 10		MIRU 16		QUB-11b		ETR-A		MIRU 26		MIRU 31		QUB-26	
	H[b]	A[c]	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A
1						1														
2			21		100	98	100			5	7	9				1	3		3	6
3	7		79			1			100	92	41	10				1	90	85		3
4				100				100		3	52	81	31	39		14	3	15	52	80
5	83	100											69	61	100	79	3		45	11
6	10															5				

[a] Number of tandem repeats (TR) copies.
[b] H, human. Data form present study.
[c] A, animal. Data from reference by Boniotti et al. (2009).

3.4. Contribution of isolate genotyping to *M. bovis* epidemiology in humans

One of the major issues of *M. bovis* epidemiology in a bovine TB-free setting is the definition of the source(s) of infection and the route(s) of transmission to humans. In theory, comparison of strain-specific molecular profiles between animal and human isolates should provide useful information on this issue. As animal strains isolated in the study region were not available for typing, we were able to compare the genetic profiles of our human *M. bovis* isolates from Tuscany with those of animal isolates, recently reported by Boniotti et al. (2009), mainly from northern Italy.

The ST482/SB0120 spoligotype, detected in 63.8% human isolates in Tuscany, was also prevalent in animals in northern Italy, accounting for 54.6% of infected herds (Boniotti et al., 2009); this spoligotype was also prevalent in animals in France (Haddad et al., 2001), Germany (Kubica et al., 2003), and Spain (Aranaz et al., 1996); it has been isolated in many other countries but apparently never in the United Kingdom or Ireland. In the very few reports on *M. bovis* isolates from humans, the ST482/SB0120 spoligotype was prevalent in Germany (ca. 20%) (Kubica et al., 2003), but rarely found in the United Kingdom (1%) (Gibson et al., 2004). Other spoligotypes detected in our human survey, besides ST482/SB0120, were reported among the animal isolates of the study of Boniotti et al. (2009), i.e., SIT665/SB0134, found in 5.8% of animal isolates, SB0934 in 1.3% and SB0927, SB0950, SB0867 found in less than 1% of animal isolates. These results confirm the concept that *M. bovis* strains by spoligotype in animals parallel those infecting humans in the same country.

Comparison of the VNTR data between our study and Boniotti's paper led to similar results; in fact, when we compared the frequency of VNTR alleles in detected 10 VNTR loci that were assayed in SIT482/SB0120 human and animal isolates (Table 3), we observed that the prevalence of the VNTR alleles was conserved in human and animal isolates in most VNTR loci (i.e., VNTR 43, MIRU 40, MIRU 16, QUB-11b, ETR-A, MIRU 26, MIRU 31, QUB-26) or that prevalent VNTR alleles differed only by 1 or 2 TR copies (MIRU 04, MIRU 10).

The spoligotype and VNTR molecular identities or similarities between human and animal isolates reported above further support the existence of a close link between human *M. bovis* TB cases and animal TB, although the region of our survey, Tuscany, has achieved the bovine TB-free status. Unfortunately, epidemiological and personal data of the study patients, due to the long time of strain collection, are incomplete to draw conclusions; however, a previous paper of ours, describing a well-characterized set of *M. bovis* TB human cases (17 cases) that occurred between 2002 and 2005 in Tuscany, reported that most Italian-born patients (11 out of 17) were 33–93-year-old (mean 69), which likely indicates, at least in most cases, a reactivation of a *M. bovis* infection acquired long time before, possibly before the introduction of the bovine TB eradication program measures; the 6 foreign-born patients with *M. bovis* TB, on the other hand, were young people (mean, 28; range 21–38-year-old), thus indicating a recent infection, likely acquired in the country of origin prior to entry to Italy (Lari et al., 2009).

4. Conclusions

Our results confirm the usefulness of VNTR analysis as major typing system for the study of the genetic diversity of *M. bovis* isolates and bovine TB epidemiology. A number of papers have addressed the relevance of the VNTR typing of *M. bovis* isolates and various sets of VNTR loci, differing in number and combination, have been proposed for the optimal resolution of *M. bovis* isolates of animal origin (Hilty et al., 2005; Allix et al., 2006; Boniotti et al., 2009), but studies on the performance of the VNTR analysis for *M. bovis* human isolates are limited (Lari et al., 2006; Ojo et al., 2008). In our setting, although only 8 loci (VNTR 43, MIRU 04, QUB-11b, ETR-A, VNTR 47, MIRU 31, QUB-26 and VNTR 53) out of the 15 tested finally provided a satisfactory resolution, VNTR analysis proved to be useful for an efficient discrimination among *M. bovis* human isolates, especially in combination with spoligotyping and occasionally integrated by additional VNTR loci when it was necessary to confirm clustered isolates. In fact, the large majority of *M. bovis* human isolates showed individual molecular profiles, although genetically closely related, and, apart the BCG isolates that clearly were subjected to a different phylogeny, only 1 cluster of identical *M. bovis* isolates was detected, which supports the possibility of a bovine TB outbreak in the study geographic area that would need to be investigated by an appropriate conventional epidemiology survey.

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