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Molecular Analysis of Clinical Isolates of *Mycobacterium bovis* Recovered from Humans in Italy[▽]

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In order to achieve a better knowledge of *Mycobacterium bovis* epidemiology in Italy, 42 clinical isolates from humans were genotyped. Predominant molecular patterns were found in one cluster of 15 isolates sharing spoligotype (ST482), variable-number tandem repeat (VNTR), and IS*6110*-based restriction fragment length polymorphism (one 1.9-kb band) profiles and in two clusters of 6 and 3 *Mycobacterium bovis* BCG isolates differing by one VNTR character. The remaining 18 isolates yielded unique profiles. Our results confirm the potential utility of spoligotyping and VNTR typing as a major typing system of *M. bovis* isolates.

Mycobacterium bovis can cause tuberculosis in a wide range of domestic and wild animals. The organism can also infect humans, producing a disease indistinguishable from that caused by *Mycobacterium tuberculosis*. In many industrialized countries, *M. bovis* has been eradicated from cattle or reduced to very low levels. In Italy as well as in other western European countries, only a few bacteriologically proven human tuberculosis cases have been attributed to *M. bovis* (12, 17, 26), but in developing countries *M. bovis* is still a cause of concern for animals and humans (5, 8). *M. bovis* is transmitted within and between domestic and wild animals as well as from animals to humans or vice versa (19). Contaminated food (especially milk) or direct contact with infected animals is considered to be the primary route by which *M. bovis* infects humans (9). Direct human-to-human transmission seems to be rare and has been confirmed mainly among immunocompromised patients (2, 19).

Molecular typing of isolates has become a valuable tool in the study of *M. bovis* epidemiology, allowing investigators to detect outbreaks and achieve better knowledge of transmission and increasing incidence of infection. However, only very few molecular surveys of human populations have been reported (5, 12, 17, 26), and no studies have been reported from Italy. This study, as a contribution to the knowledge of *M. bovis* molecular epidemiology in Italy, examines the molecular characteristics of 42 *M. bovis* clinical isolates collected from 34 Italian-born and 8 foreign-born tuberculosis patients hospitalized in Tuscany, Italy, between 1990 and 2005. A total of 13 isolates were from human immunodeficiency virus-negative patients; human immunodeficiency virus serology was not known for 29 patients. The *M. bovis* isolates were identified by the use of DNA molecular probes specific for the *M. tuberculosis* complex (Accuprobe; Gen-Probe) and by determining that niacin production and nitrate reductase activity were both negative. The isolates were typed by molecular techniques including (i) the commercial GenoType MTBC assay (Hain Lifescience GmbH, Nehren, Germany), (ii) high-performance liquid chromatography (HPLC) analysis of cell wall mycolic acids, (iii) spoligotyping, (iv) IS*6110*-based restriction fragment length polymorphism (IS*6110*-RFLP), and (v) variable-number tandem repeat (VNTR) typing. The overall molecular results are reported in Table 1.

GenoType MTBC assay. The GenoType MTBC assay targets fragments of the *rpoB* gene; the RD1 region of *M. tuberculosis* and *M. bovis*, which is absent from *M. bovis* bacillus Calmette-Guérin (BCG) (13); and the *gyrB* polymorphism, characterized by mutations suitable to differentiate most of the species belonging to the *M. tuberculosis* complex (20). All the isolates hybridized with the *rpoB* probe, thus confirming that they belonged to the *M. tuberculosis* complex. The results demonstrate that 41 strains had *gyrB* mutations typical of *M. bovis* and BCG; 9 of these strains lacked RD1 and were identified as *M. bovis* BCG. One isolate presenting RD1 had the *gyrB* mutations typical of *Mycobacterium caprae*.

HPLC. HPLC analysis of cell wall mycolic acids, performed according to the work of Butler et al. (4), yielded the specific six-clustered BCG profile for the nine isolates identified as *M. bovis* BCG by the GenoType MTBC assay. The *M. bovis* isolates, including the *M. bovis*/*M. caprae* isolate, yielded the four-clustered *M. tuberculosis* profile (10).

Spoligotyping. The spoligotyping (spacer oligonucleotide typing) technique, which probes the variability of spacer sequences interspersed with direct repeat (DR) sequences in the polymorphic chromosomal locus DR, was performed by use of the standard spoligotype assay described by Kamerbeek et al. (15). Spoligotyping detected 12 individual patterns that were assigned to one of the share types (ST) contained in the SpolDB4 (3) and the Mbovis.org (http://www.Mbovis.org) databases (the ST designation of the SpolDB4 database is used throughout the text). The prevalent spoligotype was ST482, detected in 29 isolates (69.0%). This spoligotype was found in all the *M. bovis* BCG isolates as well as in the reference strains *M. bovis* BCG Pasteur and *M. bovis* BCG Tice (data not shown). The "BCG-like" strain ST482 has been shown to be

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TABLE 1. Molecular characteristics of *M. bovis* clinical isolates from humans

No. of isolates	GenoType test		HPLC pattern[c]	Spoligotyping			IS*6110*-RFLP fingerprint[f]	VNTR-MIRU profile[g]
	RD1[a]	*gyrB*[b]		Binary pattern[d]	Designation by[e]: SpolDB4	Mbovis.org		
6	-	b	BCG		ST482	SB0120		22'2324253322
3	-	b	BCG		ST482	SB0120		23'2324253322
15	+	b	MTC		ST482	SB0120		232324252322
1	+	b	MTC		ST482	SB0120	no band	232324252322
1	+	b	MTC		ST482	SB0120		232324253322
1	+	b	MTC		ST482	SB0120		232324252522
1	+	b	MTC		ST482	SB0120		232324252222
1	+	b/c	MTC		ST482	SB0120		232324252422
1	+	b	MTC		ST820	SB0856		232324252322
1	+	b	MTC		ST820	SB0856	no band	232324252322
1	+	b	MTC		ST1932	SB0950		232324252322
1	+	b	MTC		ST1932	SB0950		232324252222
1	+	b	MTC		ST1842	SB1060		232324252322
1	+	b	MTC		ST977	SB0867		232324252322
1	+	b	MTC		ST672	SB0269		232224253322
1	+	b	MTC		ST665	SB0134		232324242422
1	+	b	ND		ST0	ND		232324252322
1	+	b	MTC		ST0	ND		232324252322
1	+	b	MTC		ST0	ND		232324252322
1	+	b	MTC		ST0	ND		232324253322
1	+	b	MTC		ST0	ND		232424252323

[a] +, RD1 region present; −, RD1 region absent.
[b] *gyrB* polymorphism by GenoType test: b, *M. bovis*/BCG-specific; b/c, *M. bovis*/*M. caprae* intermediate.
[c] HPLC pattern: BCG, BCG type; MTC, *M. tuberculosis* complex type; ND, not done.
[d] Symbols: ■, presence of the specific spacer at positions 1 to 43 in the DR locus; □, absence of the specific spaces at positions 1 to 43 in the DR locus.
[e] Spoligotype designation was assigned according the definition in SpolDB4 and in the Mbovis.org databases. ND, not described.
[f] IS*6110*-RFLP fingerprints were analyzed by the Gelcompar 4.1 software package (Applied Maths, Kortrijk, Belgium).
[g] VNTR-MIRU profile is expressed as a string of 12 numbers, each representing the number of tandem repeats at a given VNTR position (see text for more details).

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

No. of TR copies[a]	No. of isolates at VNTR-MIRU locus											
	2	4	10	16	20	23	24	26	27	31	39	40
0												
1												
2	42		42	1	42		42		30	2	42	41
2′		6										
3		33		40					12	37		1
3′		3										
4				1		42		1		2		
5								41		1		
6												
7												
8												
Allelic diversity[b]	0.00	0.34	0.00	0.07	0.00	0.00	0.00	0.02	0.39	0.20	0.00	0.02

[a] TR, tandem repeat. The symbol ′ indicates alleles devoid of 53-bp sequences at the 3′ terminus of the MIRU locus.
[b] Allelic diversity (h) was calculated using the equation $h = 1 - \Sigma x_i^2 [n/(n-1)]$, where n is the number of isolates and x_i the frequency of the ith allele at the locus (23).

prevalent in animals in Italy (24), France (14), Germany (17), and Spain (1) and 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 spoligotype was prevalent in Germany (ca. 20%) (17) but rarely found in the United Kingdom (1%) (12), thus paralleling the spoligotype distribution in animals. Spoligotypes ST820 and ST1932, found in two isolates, and spoligotypes ST1842, ST977, ST672, and ST665, found in individual isolates, have previously been detected in several different geographical areas among humans (3) and animals (6, 14). Five individual spoligotypes detected among our human isolates had not been described previously; these isolates, generally referred to as "orphan," are labeled as ST0, according to the rules of the SpolDB4 database.

***IS6110*-RFLP.** The IS*6110*-RFLP assay was performed according to the standardized method described by van Embden et al. (27). IS*6110*-RFLP analysis is known to provide the best discrimination for *M. tuberculosis* isolates but is considered poorly discriminatory for *M. bovis* isolates from cattle, which usually have a single copy of IS*6110* (7, 16). In the present study, the 42 isolates yielded four distinct IS*6110* banding patterns, each consisting of one or two IS*6110* bands, as expected. Similar to the reference BCG Pasteur and Tice strains, 35 isolates, including all the BCG isolates and 26 *M. bovis* isolates, showed a single restriction fragment of 1.9 kb; 2 *M. bovis* isolates yielded a single band slightly smaller than 1.9 kb. The two-band profile was found for two isolates yielding the 1.9-kb band and an additional smaller band as well as for one isolate yielding the 1.9-kb band and a larger band. Two *M. bovis* isolates did not show any IS*6110* restriction fragment.

VNTR typing. VNTR typing is aimed at displaying variations in the copy number of tandem repeats located in mycobacterial interspersed repetitive units (MIRU) in the chromosome. This technique has been successfully used to differentiate *M. tuberculosis* strains (25) and to discriminate between *M. bovis* strains (11, 21). VNTR typing was performed by PCR amplification of 12 MIRU loci (i.e., loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40) as described by Supply et al. (25). 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, California), and the numbers of repetitive units were calculated.

VNTR-MIRU analysis yielded 10 different profiles among the isolates. The nine *M. bovis* BCG isolates showed, at locus 4, two alleles not found in any other isolate of the collection: in particular, six isolates, as well as the reference strains BCG Pasteur and Tice (not shown in Table 1), displayed two copies of a 77-bp repeat unit specifically located at locus 4 but not a 53-bp sequence that is commonly found at the 3′ terminus in all the strains of the *M. tuberculosis* complex. This allele is usually indicated as 2′, as the symbol ′ designates alleles devoid of the 53-bp repeat (18). Similarly, the other three BCG isolates showed, at locus 4, three copies of the 77-bp repeat unit, indicated as 3′, and were devoid of the terminal 53-bp sequence. As to the *M. bovis* isolates, the prevalent VNTR-MIRU profile was 232324252322; this profile was found for 24 isolates, 16 of ST482, 2 of ST820, and the remaining ones of individual spoligotypes or orphan. Other VNTR-MIRU profiles, shared by two isolates, were 232324252222 (ST482 and ST1932) and 232324253322 (ST482 and orphan).

These results indicate that, contrary to what is seen for *M. tuberculosis* isolates, for which the MIRU profile is generally considered an individual marker equivalent to the IS*6110*-RFLP profile, VNTR typing of *M. bovis* isolates is poorly discriminatory. In fact, the allelic diversity (h) of the 12 MIRU loci for the 42 *M. bovis* strains, calculated using the equation $h = 1 - \Sigma x_i^2 [n/(n-1)]$, where n is the number of isolates and x_i the frequency of the ith allele at the locus (23), was null or extremely low for most loci (Table 2). In particular, six loci (i.e., 2, 10, 20, 23, 24, and 39) displayed no diversity ($h = 0$), and loci 16, 26, and 40 were poorly discriminative ($0.02 < h < 0.07$). Thus, only loci 27, 4, and 31 showed a satisfactory discriminatory power ($h \geq 0.20$).

Taken together, the results of this study show that a total of 21 unique profiles, differing by at least one molecular character, were found among the *M. bovis* isolates from tuberculosis patients (each profile is shown in one row of Table 1). Three clusters were detected: the predominant molecular pattern was

found in one cluster of 15 isolates sharing spoligotype, VNTR-MIRU loci, and IS*6110*-RFLP profile. The two other clusters included six and three *M. bovis* BCG isolates that differed by one VNTR-MIRU locus. The remaining 18 isolates yielded unique profiles.

The results of this study, which provides a first description of the population structure of *M. bovis* in Italy, will be useful for further molecular surveys aimed at achieving a better knowledge of moving and expanding strains in our country. Moreover, the results confirm the potential utility of spoligotyping and VNTR-MIRU typing, although with the limits outlined above, as a major typing system for *M. bovis* isolates. On this last subject, we agree with the statement of Roring and coworkers (21, 22) that for *M. bovis* typing the number and combination of informative VNTR markers selected for epidemiological studies need to be determined empirically with reference to locally prevalent strains.

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