Evaluation of the Epidemiological Relevance of Variable-Number Tandem-Repeat Genotyping of *Mycobacterium bovis* and Comparison of the Method with IS*6110* Restriction Fragment Length Polymorphism Analysis and Spoligotyping†

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Sources of *Mycobacterium bovis* contamination remain unclear for many cases of animal and human disease. A major limitation is the lack of sufficiently informative or epidemiologically well evaluated molecular methods for typing. Here, we report an evaluation of a high-throughput method based on 29 mycobacterial interspersed repetitive unit–variable-number tandem-repeat (MIRU-VNTR) loci to genotype 127 *M. bovis* isolates from cattle from 77 different Belgian farms, representative of a nationwide collection obtained from 1995 to 2003. MIRU-VNTR stability was demonstrated by analyzing a series of 74 isolates in total, obtained from different animals from a single farm or from different farms with an identified epidemiological link. The genotyping results and the genotypic diversity (*h*) were compared with those obtained by IS*6110* restriction fragment length polymorphism (RFLP) analysis and spoligotyping. Among 68 isolates with no known epidemiological link, MIRU-VNTR typing discriminated better than either RFLP analysis or spoligotyping, with isolates taken individually (32 versus 16 and 17 genotypes; *h* = 0.91 versus 0.73 and 0.85, respectively) or in combination (32 versus 28 genotypes; *h* = 0.91 versus 0.92). Maximal resolution was already achieved with a subset of 9 loci. The observed congruence of the genetic relationships based on IS*6110* RFLP analysis, spoligotyping, and MIRU-VNTR markers is consistent with a clonal population structure of *M. bovis*. These results support MIRU-VNTR typing as a convenient and discriminatory technique for analysis of the population structure of *M. bovis* in much greater detail and for addressing some still unresolved issues in the epidemiology of the pathogen.

Mycobacterium bovis is the causative agent of tuberculosis in bovines. In addition to cattle, this pathogen has an exceptionally wide host range, extending to goats, cats, dogs, pigs, lions, deer, nonhuman primates, and humans. Many susceptible species, including humans, are putative spillover hosts, in which infection is not confined. Some countries, such as Great Britain and Ireland, are currently experiencing an exponential increase in the incidence of bovine tuberculosis. Moreover, *M. bovis* infection is highly prevalent in farmed animals in developing countries (5). Many of these countries have endemically infected wildlife, which is suspected of providing a reservoir for farmed animals. Under such conditions, eradication is not feasible, and control measures must be applied indefinitely (20). Therefore, *M. bovis* is a source of important economic damage and a potential threat to human health in many countries.

A few studies have estimated the proportion of *M. bovis* infection in human tuberculosis cases to be in the range of 0.3 to 1.5% in developed countries (5–7, 10, 24). This proportion may be higher in some developing countries, although little information is available (5). Cases of human tuberculosis with *M. bovis* are mainly associated with the consumption of unpasteurized dairy products or close contact with infected animals (3, 10). Therefore, these are most often suspected of being of zoonotic origin. Cases of interhuman transmission have only rarely been documented (10, 12, 19). However, the exact source of infection remains undetermined in most cases. Likewise, direct transmission between wildlife and farmed animals has not been demonstrated yet under natural conditions. Nev-

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ertheless, it has been demonstrated that experimentally infected deer can transmit *M. bovis* to cattle through the sharing of feed (22).

One main limitation results from the lack of sufficiently informative molecular methods for typing *M. bovis*. In contrast to *Mycobacterium tuberculosis*, restriction fragment length polymorphism typing using IS*6110* (IS*6110* RFLP) provides only limited discrimination among *M. bovis* isolates, since *M. bovis* strains usually only have a single or a few IS*6110* copies. PCR-based spoligotyping, targeting the direct repeat region, also suffers from the same limitation. Analysis of multiple genomic regions that contain variable-number tandem repeats (VNTRs) of different families of genetic elements called mycobacterial interspersed repetitive units (MIRUs) (9, 18, 25, 27, 29, 31) has been recently proposed as an alternative tool for molecular epidemiological studies of *M. bovis*. In a sample of 47 isolates from Northern Ireland, these markers were shown to be more discriminatory than spoligotyping (26). However, the epidemiological interpretation of MIRU-VNTR genotyping data is difficult to assess accurately from studies in such a setting. Because of its high incidence, cattle movements, and the existence of potential environmental reservoirs (i.e., badgers), independent sources of infection may occur within given herds, and unsuspected transmission chains might often exist between farms. Therefore, it is difficult to evaluate the actual capacity to distinguish epidemiologically unrelated isolates and the stability of the markers among epidemiologically related isolates. Moreover, the performances of MIRU-VNTR typing data were not compared with those of IS*6110* RFLP fingerprinting in that study.

Here, we have evaluated the clonal stability, epidemiological significance, and discrimination of 29 MIRU-VNTR loci (9, 18, 25, 27, 29, 31) using a sample of 127 *M. bovis* isolates from Belgium. This country has an extremely low incidence of bovine tuberculosis (0.02% of infected herds in 2003 and 2004) and no known environmental reservoir of *M. bovis*. The stability of the markers necessary to trace transmission chains over time was tested using several sets of isolates with identical spoligotypes and IS*6110* fingerprints from a single farm and by investigating available cattle tracing data for clustered isolates obtained from different farms. The discriminatory power was evaluated using isolates representative of all of the different spoligotypes, IS*6110* fingerprints available from a nationwide collection, and isolates with identical spoligotypes and IS*6110* fingerprints from different farms with no obvious epidemiological link. Finally, the congruence of the genetic relationships as defined by IS*6110* RFLP, spoligotyping, and MIRU-VNTR typing was analyzed.

MATERIALS AND METHODS

Control of bovine tuberculosis in Belgium and epidemiological investigation. The control of bovine tuberculosis is currently based on skin testing in the context of animal contact tracing and trading. In addition, systematic postmortem examinations at slaughterhouses and individual postmortem examinations at the regional veterinary laboratories are performed. When a suspected lesion is identified, a sample is sent to the reference laboratory for analysis. Subsequently, all animals from the herd of origin are skin tested, and a complete epidemiological investigation is carried out, following standardized epidemiological procedures. The aim of this investigation is to determine the origin of the outbreak and to identify all farms in contact with infected animals or materials. This identification is possible by consulting SANITEL (Belgian databank for the registration and movement of cattle).

Study collection. One hundred twenty-seven *M. bovis* isolates originating from cases of bovine tuberculosis diagnosed in 77 herds in Belgium during the period 1995 to 2003 were included in this study. The isolates were chosen among 451 samples from 204 herds characterized by IS*6110* RFLP typing and spoligotyping methods from a total of 503 animal tuberculosis cases with positive cultures identified on 220 Belgian farms over this 9-year period. This collection is maintained at the Belgian National Reference Laboratory (CERVA-CODA, Brussels, Belgium). The isolates typed by the MIRU-VNTR method were chosen and grouped into three panels, named panels 1, 2, and 3, according to three analytical criteria (see Results and Table S in the supplemental material). Among these 127 samples, 26 were the result of pooled tissue samples from different animals from a single herd. In these cases, biopsy specimens of 2 to 33 different animals (usually from 2 to 5) from the same farm collected at the same time were pooled.

Bacteriology. Samples from animals suspected of suffering from tuberculosis were pooled, homogenized with sterile saline solution, and decontaminated with 5% oxalic acid for 20 to 30 min at 37°C (21). After centrifugation at $1,000 \times g$ for 20 min, isolates were cultured on Coletsos solid medium (Bio-Rad, Belgium) at 37°C. Bacteriological identification of *M. bovis* was based on acid-fast staining, preferential growth at 37°C and 42°C, the nitrate reductase test (Becton Dickinson, Belgium), and the tiophene-2-carboxilic acid hydrazide assay (Biomérieux, Belgium).

DNA extraction. Mycobacterial strains were grown in Middlebrook 7H9 liquid medium supplemented with oleic acid-albumin-dextrose-catalase (Becton Dickinson, Belgium), Tween 80, penicillin, and Fungizone for 3 weeks. Chromosomal DNA was isolated as described by van Embden et al. (34).

Genotyping. (i) IS*6110* RFLP analysis. DNA fingerprinting was performed as described by van Embden et al. (34). Briefly, purified mycobacterial DNA was digested with PvuII, separated on an agarose gel, transferred by Southern blotting to a nylon membrane, and hybridized with a peroxidase-labeled 245-bp IS*6110* probe. Hybridizing restriction fragments were visualized using the ECL detection system (Amersham Biosciences, Belgium).

(ii) Spoligotyping. The spoligotyping method was used as described by Kamerbeek et al. (16). PCR amplification of the direct repeat locus was performed on heat-treated cell suspensions. Biotin-labeled PCR products were detected by hybridization onto a spoligotyping membrane (Isogen Bioscience BV, Maarssen, The Netherlands). Purified sterile water and clinical isolates of *M. tuberculosis* and *M. bovis* were included as controls in every batch of tests. The results were recorded in SB (spoligotype bovis) code, followed by a field of 4 digits as defined on the *Mycobacterium bovis* Spoligotype Database website (www.Mbovis.org).

(iii) MIRU-VNTR. MIRU-VNTR analysis was done by amplification of 29 genomic loci in 9 different multiplex PCRs and 2 different simplex PCRs with the previously described primers (9, 18, 25, 27, 29, 30) (Table 1). Multiplex PCR mixtures were prepared as follows, using 96-well plates and the Hotstart *Taq* DNA polymerase kit (QIAGEN, Benelux B.V.). Three nanograms of DNA was added to a final volume of 30 μl containing 0.1 μl of DNA polymerase (1 U); 6 μl of Q solution; 0.2 mM (each) dATP, dCTP, dGTP, dTTP (Fermentas GmbH, Germany); 3 μl of PCR buffer; 0.4 μM concentrations of each primer (Applied Biosystems, Belgium); and different concentrations of MgCl$_2$, as shown in Table 1. For each multiplex mixture, one primer from each oligonucleotide pair was tagged with a different fluorescent dye. The thermocycler programs were identical for all of the reactions. The PCRs were carried out using a GeneAmp PCR system 2700 (Applied Biosystems, Belgium) starting with a denaturing step of 15 min at 95°C, followed by 40 (VNTR 3232 and 3336) or 30 (all other loci) cycles of 1 min at 94°C, 1 min at 59°C, and 1 min, 30 s at 72°C. Two microliters of PCR products was mixed with formamide and a MapMarker1000 ladder (23 fragments ranging from 50 to 1,000 bp; Eurogentec, Belgium). DNA fragments were separated by capillary electrophoresis using an ABI Prism 3100-Avant genetic analyzer. VNTR numbers were scored based on amplicon sizes using Genescan and customized Genotyper software packages (Applied Biosystems, Belgium).

(iv) RD4 analysis. PCR analysis of the RD4 deletion region, defining classical *M. bovis* strains, was performed using primers and conditions, as described by Brosch et al. (4).

Allelic and genotypic diversity. The allelic diversity of each VNTR locus was calculated using the following equation: $h = 1 - \Sigma x_i^2 [(n/n - 1)]$, where n is the number of tested isolates and x_i is the frequency of the ith allele. Genotypic diversity was used as a measure of the discriminatory power of the three genotyping methods. It was defined as $1 - \Sigma x_i^2 [(n/n - 1)]$, where n is the total number of genotypes obtained and x_i is the frequency of the ith genotype.

Genetic relationship analysis. IS*6110* RFLP patterns were recorded and analyzed using the Bionumerics package (Applied Maths, St-Martin-Latem, Belgium) as fingerprint data. The Dice coefficient was used to plot dendrograms

TABLE 1. Primer sequences and MgCl$_2$ concentrations of the different PCR mixes

Multiplex	Locus	Alias	MIRU-VNTR length (bp)	MgCl$_2$ concn (mM)[b]	PCR primer pair (5′–3′, with labeling indicated)[a]
Mix A	MIRU 4	VNTR 580 ETR-D	77		GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGTCAGC
	MIRU 26	VNTR 2996	51	3	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)
	MIRU 40	VNTR 802	54		GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
Mix B	MIRU 10	VNTR 960	53		GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT (FAM)
	MIRU 16	VNTR 1644	53	2	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (VIC)
	MIRU 31	VNTR 3192 ETR-E	53		ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)
Mix C	MIRU 2	VNTR 154	53		TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)
	MIRU 23	VNTR 2531	53	2.5	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGGTTCGCCCTTTTGTC
	MIRU 39	VNTR 4348	53		CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT (NED)
Mix D	MIRU 20	VNTR 2059	77		TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA
	MIRU 24	VNTR 2687	54	1.5	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)
	MIRU 27	VNTR 3007 QUB5	53		TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)
Mix E	VNTR 2347		57		GCCAGCCGCCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC
	ETR-B	VNTR 2461	57	1.5	ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC
	VNTR 3171		54		GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC
Mix F	VNTR 0424		51		CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC (FAM)
	ETR-C	VNTR 0577	58	1.5	CGAGAGTGGCAGTGGCGGTTATCT (VIC) AATGACTTGAACGCGCAAATTGTGA
	VNTR 1895	QUB 1895	57		GTGAGCAGGCCCAGCAGACT (NED) CCACGAAATGTTCAAACACCTCAAT
Mix G	VNTR 2401		58		CTTGAAGCCCCGGTCTCATCTGT (FAM) ACTTGAACCCCCACGCCCATTAGTA
	VNTR 3690		58	3.0	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
	VNTR 4156	QUB 4156	59		TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (NED)
Mix I	QUB 11a	VNTR 2163a	69		CCCATCCCGCTTAGCACATTCGTA TTCAGGGGGGATCCGGGA (FAM)
	QUB 26	VNTR 4052	111	1.5	AACGCTCAGCTGTCGGAT (VIC) GGCCAGGTCCTTCCCGAT
	ETR-A	VNTR 2165	75		AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT
Mix J	QUB 11b	VNTR 2163b	69		CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT (FAM)
	VNTR 1955		57	1.5	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA
	VNTR 1982	QUB 18	78		CCGGAATCTGCAATGGCGGCAAATTAAAAG TGATCTGACTCTGCCGCCGCTGCAAATA (NED)
Individual	VNTR 3232	QUB 3232	56/57	1.5	TGCCGCCATGTTTCATCAGGATTAA GCAGACGTCGTGCTCATCGATACA (FAM)
Individual	VNTR 3336	QUB 3336	59	1.5	ATCCCCGCGGTACCCATC (VIC) TTCTACGACTTCGCAACCAAGTATC

[a] Forward and reverse primers, respectively. FAM, blue dye label (6-carboxy-fluorescein); NED, yellow dye label; VIC, green dye label.
[b] Final concentration (Hotstart buffer already contains 1.5 mM MgCl$_2$).

using the unweighted pair group method with arithmetic averages (UPGMA). Spoligotyping and MIRU-VNTR profiles were recorded as character data and were analyzed using the categorical character option and UPGMA to align branch tips for better visualization in Fig. 1 and 2 or the neighbor-joining algorithm for congruence analysis in Fig. 4. Composite data sets were created to

analyze clustering resulting from a combination of the different genotyping methods.

Congruence between the genetic distances based on MIRU-VNTR types and IS*6110* fingerprints and spoligotypes was evaluated with Bionumerics, using the Pearson correlation. The g test (32) was used to evaluate the statistical signifi-

FIG. 1. IS6110 RFLP, spoligotyping, and MIRU-VNTR patterns of isolates obtained from different animals from a single farm. The dendrogram at the left is based on MIRU-VNTR profiles and was built by using the UPGMA algorithm, as described in Materials and Methods. Double alleles (4 and 7) detected in locus 3232 of two pooled samples (02011T1 and 02011T2) are boxed. a, a number (Nb) of >1 indicates a sample obtained by pooling tissues of n different animals from the same herd before mycobacteriological culture.

cance of this congruence. This test takes random association as a null hypothesis and was done by evaluating the significance of the correlation between the genetic distances inferred from the above methods for any possible pair of isolates, with a nonparametric Mantel test based on a Monte Carlo simulation with 10^4 iterations.

RESULTS

Selection of isolates. One hundred twenty-seven *M. bovis* isolates originating from cases of bovine tuberculosis diagnosed on 77 Belgian farms between 1995 and 2003 were included in this study. The isolates were selected from a nationwide collection of 451 isolates collected between 1995 and 2003 at the Belgian National Reference Laboratory, representing 89.6% of all animal tuberculosis cases with a positive culture identified in Belgium over this 9-year period. The isolates were chosen according to three criteria: panel 1 included 13 series of 2 to 6 isolates collected from different animals coming from a single farm, panel 2 included 5 series of 2 to 11 isolates obtained from different farms but with a defined epidemiological link, while panel 3 included isolates representative of all the different spoligotypes and/or IS*6110* fingerprints available in the nationwide collection as well as isolates with identical spoligotypes and IS*6110* fingerprints from different farms without obvious epidemiological links. With this system, some isolates were represented in more than one panel, as for instance, a sample representative of a defined spoligotype (panel 3) could be found with other isolates from the same farm (panel 1) and also with isolates from another epidemiologically linked farm (panel 2) (see Table S in the supplemental material).

Stability of MIRU-VNTR loci. (i) Multiple isolates from individual farms (panel 1). The stability of the 29 MIRU-VNTR loci was assessed by analyzing 13 groups including 57 *M. bovis* isolates in total (panel 1) (Fig. 1). Each group included between 2 and 6 isolates with identical IS*6110* RFLP patterns and spoligotypes, obtained from different animals from a single farm over periods of up to 4 years. We postulated that the cows within these groups were probably infected from a single source and thus involved a single transmission chain. Consistent with this hypothesis, the 29 loci remained unchanged within all groups except one. In this group, the simultaneous occurrence of two different alleles (4 and 7) was clearly detected in locus 3232 of two samples (02011T1 and 02011T2), while the two other isolates of the group displayed a single allele 4 in this locus. Samples 02011T1 and 02011T2 were each the result of a pool of tissue samples from 5 and 4 different animals, respectively. Therefore, this observation may indicate either the presence of a second strain or, alternatively, of a clonal variant in a limited number of animals in this herd. There was no such evidence of genotypic heterogeneity in any of the other pooled samples in this panel or in the other 2 panels (see below). This was consistent with complete genotypic homogeneity among all the isolates obtained from several different animals from individual farms, suggesting that such isolates share a common clonal origin in most cases.

Therefore, in accordance with our earlier hypothesis, these results indicate that the 29 loci were stable in transmission chains spanning periods of up to 4 years on at least 12 farms of the 13 examined.

(ii) Epidemiologically linked isolates from different farms (panel 2). Panel 2 included samples obtained from different farms among which epidemiological links could be clearly established by analysis of complete cattle tracing data. This panel comprised five clusters (named CE1 to CE5), within which IS*6110* RFLP and spoligotyping patterns were identical or almost identical (Fig. 2).

In cluster CE1, a link could be found between four farms and a cattle trader (Fig. 3). Farm A had a tuberculosis outbreak identified in 2000 but was suspected of having hosted diseased animals since at least 1996. The cattle trader bought animals from farmer A and sold them to farmer B. In turn, animals from farm B were transferred to farm C. One isolate (00105T) from farm A displayed a fully identical genotype by the three genotyping methods when compared to all the isolates obtained from the trader and from farms B and C. Interestingly, it was found that the cattle trader also sold animals to farm D. There was a difference in the RFLP pattern in the isolate from this farm due to an additional IS*6110* band, but the MIRU-VNTR code did not differ from the above samples (Fig. 2). Conversely, one isolate (00059) from farm A displayed a single repeat change in one MIRU-VNTR locus, together with a single band change in its IS*6110* fingerprint, compared to the other members of the cluster.

In cluster CE2, the four isolates from farms E and F all shared the same MIRU-VNTR genotype over the 29 loci, although one isolate (96171) from farm F had one less IS*6110* band than the pattern shared with the other isolate from this farm and the two isolates from farm E (Fig. 2). A direct epidemiological link was clearly established between the two farms involved, as it was found that the two farmers worked together on both farms and their animals shared the same pastures.

The other clusters showed the same profiles by the three genotyping methods (Fig. 2). In cluster CE3, the farms were epidemiologically linked through the purchase of animals (farm G to farm J) or by being neighbors (farms H, I, and J). In cluster CE4, a direct epidemiological link was established between the two farms involved, as the discovery of the outbreak on farm L was actually traced back to animals originating from farm K and led to the identification of an unsuspected outbreak on the latter farm. Likewise, a direct epidemiological link was established between the three farms in cluster CE5 because the discovery of outbreaks on farms M and O was traced back to animals originating on farm N and led to the identification of an unsuspected outbreak on this farm.

Discriminatory power and genotypic diversity (panel 3). The discriminatory power of MIRU-VNTR typing was compared to that of IS*6110* RFLP and spoligotyping by analyzing 68 isolates in panel 3 (Table 2 and Fig. 4). This panel was designed to include isolates assumed to be without epidemiological links. These isolates were representative of all the different IS*6110* fingerprints as well as of all the different spoligotypes identified in this nationwide collection. In addition, panel 3 included isolates with identical spoligotypes and IS*6110* fingerprints from different farms without obvious epidemiological links based on cattle tracing.

In this collection, different RFLP groups were named according to the IS*6110* fingerprints of the isolates, based on a code described by Rigouts et al. (23). Groups A and G included *M. bovis* isolates with a single IS*6110* band and high (8 to 11 bands) IS*6110* copy numbers, respectively. These groups
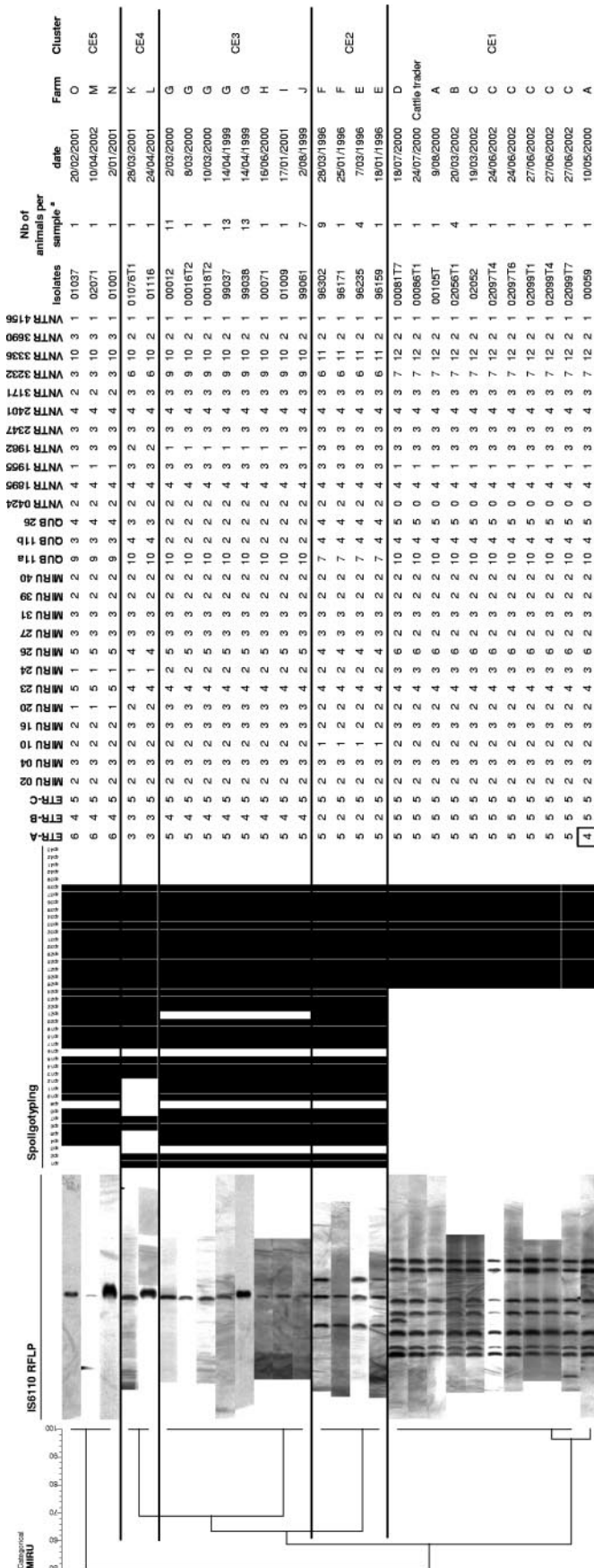
FIG. 2. IS6110 RFLP, spoligotyping, and MIRU-VNTR patterns of isolates obtained from animals from different farms included in five distinct clusters with defined epidemiological links. The dendrogram at the left is based on MIRU-VNTR profiles and was built by using the UPGMA algorithm, as described in Materials and Methods. The single repeat change in locus ETR-A of sample 00059 compared to the same locus of the other isolates of cluster CE1 is boxed. a, a number (Nb) of >1 indicates a sample obtained by pooling tissues of n different animals from the same herd before mycobacteriological culture. See text and Fig. 3 for descriptions of farms and clusters.
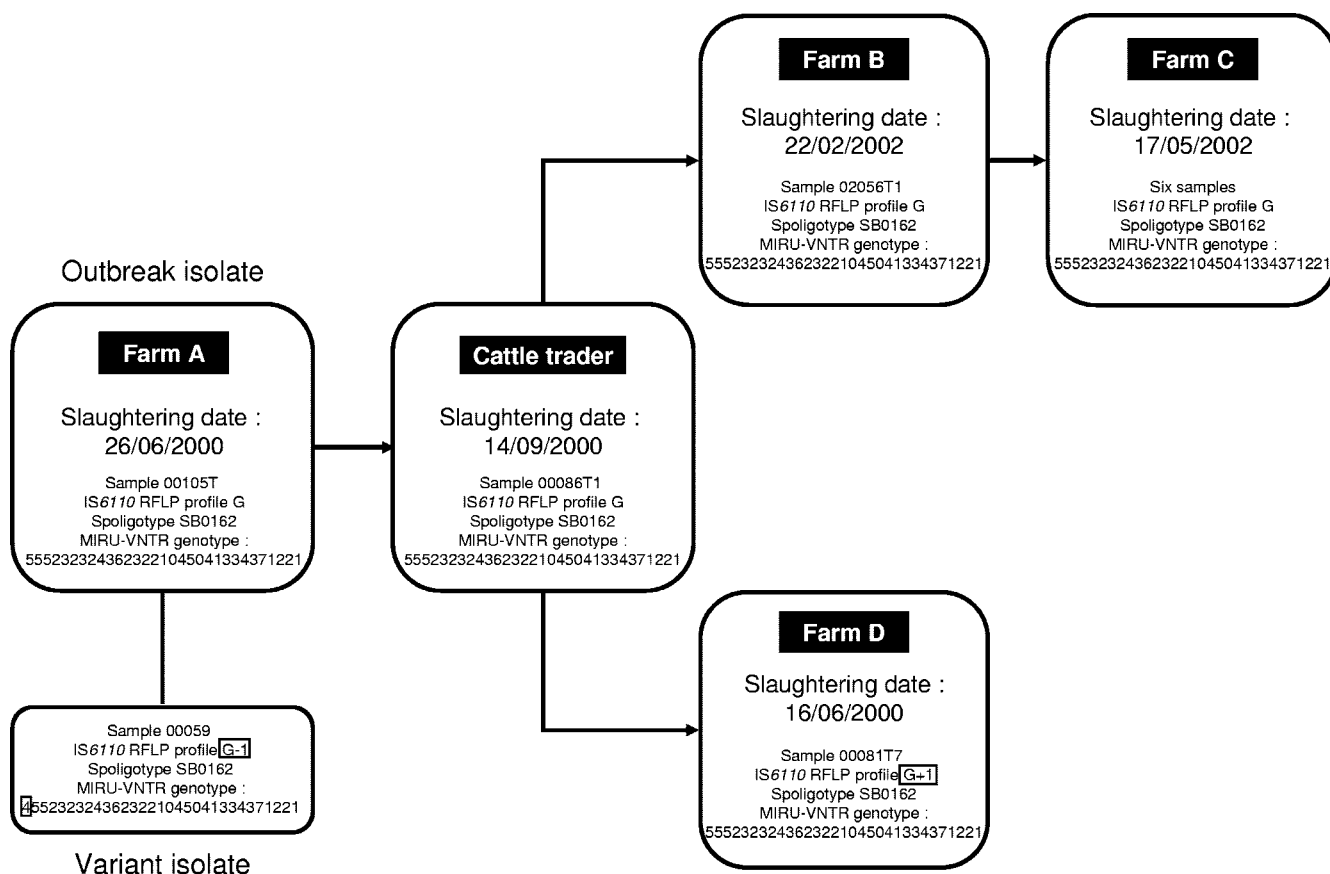
FIG. 3. Bovine tuberculosis transmission chain between different farms involving animals sold by one cattle trader. The cattle trader bought animals from farmer A and sold them to farmer B. In turn, animals from farm B were transferred to farm C. Samples from farms B and C and the cattle trader displayed completely identical genotypes (from the three genotyping methods) to that of isolate 00105T from farm A. Presumed clonal variants with a single band change in the IS*6110* RFLP pattern (see boxed RFLP profiles for isolates 00059 and 00081T7) accompanied by a single repeat change in the ETR-A locus for isolate 00059 (see boxed allele) were found on farms A and D, respectively.

were the most prevalent in Belgium with 43% and 48% of the isolates, respectively. The remaining 9% comprised five minor and distinct fingerprint groups, including isolates with intermediate numbers of IS*6110* copies, designated H (4 to 5

TABLE 2. Number of different patterns observed among the *M. bovis* isolates in panel 3 and calculated genotypic diversities generated by IS*6110* RFLP, spoligotyping, and MIRU-VNTR typing, taken alone or in combination

Technique(s)	No. of different patterns	Genotypic diversity
IS*6110* RFLP	16	0.73
Spoligotyping	17	0.85
MIRU-VNTR 29 loci	32	0.91
IS*6110* RFLP + spoligotyping	28	0.92
Spoligotyping + VNTR 29 loci	32	0.91
IS*6110* RFLP + VNTR 29 loci	37	0.95
IS*6110* RFLP + spoligotyping + VNTR 29 loci	37	0.95
Spoligotyping + VNTR 6 loci[a]	29	0.90
IS*6110* RFLP + VNTR 6 loci[a]	35	0.94
IS*6110* RFLP + spoligotyping + VNTR 6 loci[a]	35	0.94

[a] The 6 loci are VNTR 3232, ETR-B, ETR-A, MIRU-26, QUB11b, and QUB11a.

bands), J (2 to 3 bands), K (4 to 6 bands), L (3 bands), and M (4 bands).

Overall, MIRU-VNTR typing discriminated 32 different patterns in this panel, corresponding to a genotypic diversity of 0.91. In comparison, only 16 different patterns were obtained with IS*6110* RFLP and 17 patterns were obtained with spoligotyping, corresponding to genotypic diversities of 0.73 and 0.85, respectively. MIRU-VNTR typing performed even better than a combination of spoligotyping and IS*6110* RFLP, which discriminated 28 patterns (Table 2).

Spoligotypes were often distinguished by MIRU-VNTR typing. For instance, the single SB0162 spoligotype characterizing the highly prevalent RFLP group G corresponded to 5 distinct MIRU-VNTR types. In contrast, spoligotyping did not discriminate any MIRU-VNTR genotypes. Likewise, none of the MIRU-VNTR genotypes were distinguished by IS*6110* fingerprinting in RFLP group A. In contrast, isolates with no known epidemiological links within three MIRU-VNTR clusters corresponding to the RFLP groups H, J, and G with intermediate or high numbers of IS*6110* bands were distinguished by single-band differences in their respective IS*6110* fingerprints. Therefore, maximal discrimination was apparently achieved by combining MIRU-VNTR and IS*6110*
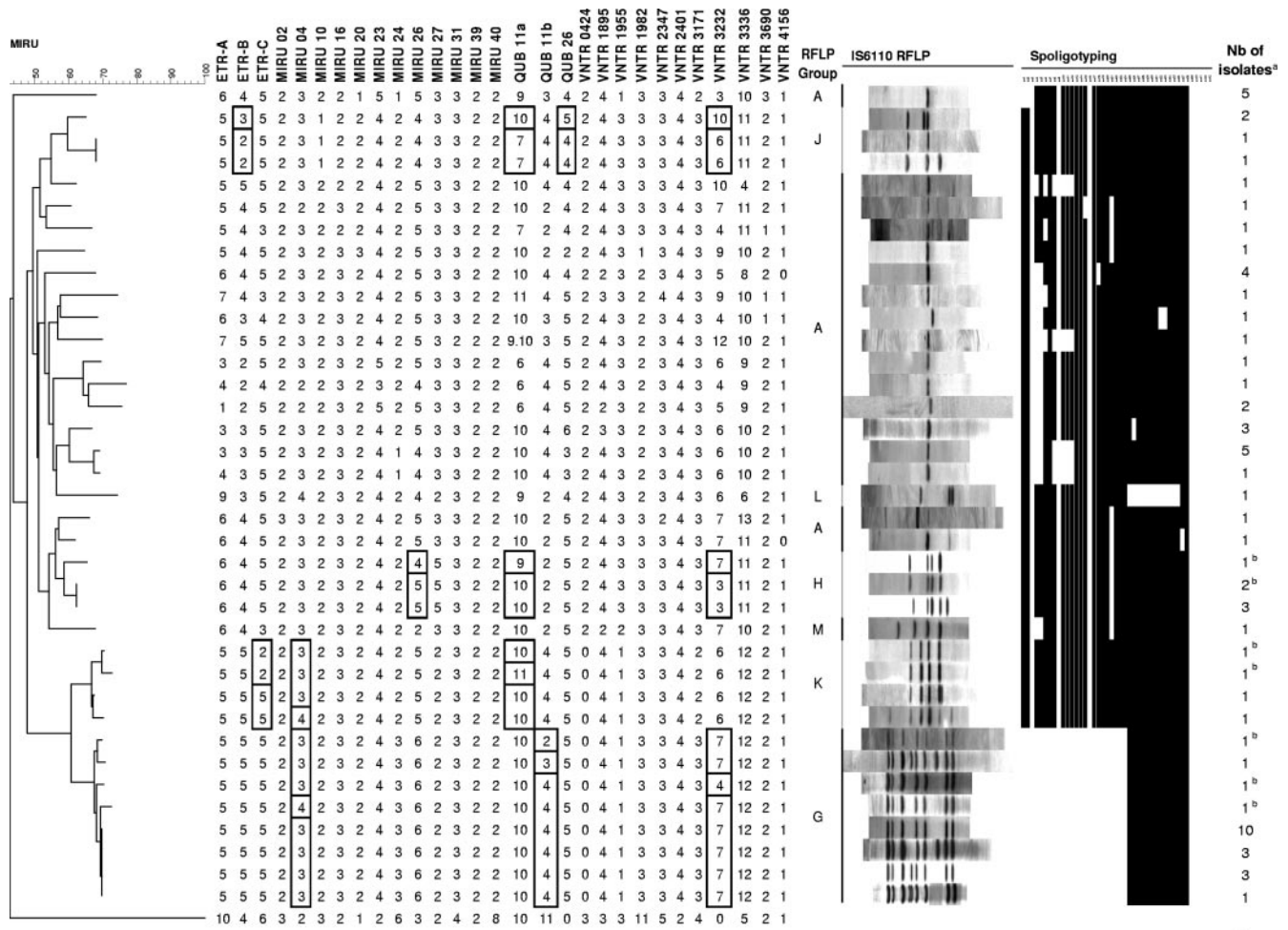
FIG. 4. Congruence of genetic relationships between MIRU-VNTR typing, IS*6110* RFLP, and spoligotyping. The dendrogram was constructed based on MIRU-VNTR genotypes using the neighbor-joining method, as described in Materials and Methods. The MIRU-VNTR tree was rooted using the *Mycobacterium canetti* clonal group C MIRU-VNTR genotype (11). The figure displays 37 different patterns among the isolates in panel 3, identified by the three genotyping methods. Boxed alleles correspond to MIRU-VNTR differences observed among samples within the G, H, J, and K lineages (see text). a, the number (Nb) of isolates with an identical genotype using the three genotyping methods. b, isolates with an identical IS*6110* fingerprint within RFLP groups which were distinguished by MIRU-VNTR typing.

RFLP typing, resulting in 37 patterns and a genotype diversity of 0.95.

Allelic diversity of VNTR loci (panel 3). Compared with each other, the 29 VNTR loci did not display equal discriminatory power in panel 3. In this test panel, MIRU 39 and 40 and VNTR 2401 displayed only a single allele, while at the other extremity VNTR 3232 displayed 8 different alleles (Table 3). Moreover, for a given locus, some alleles were clearly much more frequent than others (for instance, the allele with 10 repeats in QUB11a was encountered in 67% of the isolates, although 6 different alleles were identified in this locus). The resolution provided by each locus was thus quantified by calculating its allelic diversity, depending upon both the number and the distribution of the alleles. Allelic diversities ranged from 0.00 for MIRU 39 and 40 and VNTR 2401 to 0.76 for VNTR 3232.

We tested various combinations of loci to identify a minimal subset that provided the same resolution as the total set of 29 loci. In our test panel, the maximal resolution of the 32 patterns obtained with the 29 loci was already achieved using a subset of 9 loci (Table 4). This subset thus corresponded to the minimal combination required to achieve nonredundant discrimination. These loci all displayed an allelic diversity greater than 0.10 in panel 3. However, they were not all ranked in the top 9 in the hierarchy of allelic diversities among the 29 loci. For instance, VNTR 4156, MIRU 4, and ETR-C displayed allelic diversities of 0.12, 0.18, and 0.18, respectively, which were much lower than those of VNTR 1955 (0.50) or MIRU 24 (0.60). These three former loci were included in the minimal subset because, in some instances, one of these three markers was involved in a single-locus variation, i.e., it was the only locus out of the 29 to distinguish an isolate from other closely related isolates. In contrast, neither VNTR 1955 nor MIRU 24 was included in the minimal subset in our collection because they were never involved in single-locus variations. Therefore, the discrimination provided was always redundant with that

TABLE 3. MIRU-VNTR allelic distribution among 68 isolates in panel 3[b]

Locus	\multicolumn{15}{c}{No. of isolates with MIRU allele:}	Allelic diversity (h)
	0[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	9 and 10	
MIRU 39			68													0.00
MIRU 40			68													0.00
VNTR 2401					68											0.00
MIRU 02			67	1												0.01
MIRU 31			1	67												0.01
VNTR 2347			1	66	1											0.01
MIRU 10		4	64													0.10
VNTR 4156	5	63														0.12
MIRU 04		6	61	1												0.18
MIRU 20			4	61	3											0.18
ETR-C			2	3	2	61										0.18
VNTR 3690		3	59	6												0.23
MIRU 23			10	58												0.24
VNTR 1895				1	58	9										0.24
VNTR 3171			7	4	57											0.27
MIRU 16			12	56												0.28
VNTR 1982							4	3		8	50	2			1	0.43
QUB 11a		1	21	46												0.44
VNTR 424	25		43													0.46
QUB 11b			13	9	46											0.48
VNTR 1955		31	1	36												0.50
QUB 26			26	37		5										0.55
MIRU 27			1	6	16	42	3									0.55
MIRU 24		12	35	21												0.60
MIRU 26			1		13	33	21									0.63
ETR-A	2	9	2		33	19	2		1							0.66
ETR-B		6	13	22	27											0.69
VNTR 3336					1		1		4	4	20	12	25	1		0.74
VNTR 3232				10	4	6	17	25		2	3		1			0.76

[a] Corresponds to amplified alleles devoid of any repeat unit.
[b] The 29 loci are listed on the left, the alleles are at the top and the calculated allelic diversities on the right. Loci are presented by increasing calculated allelic diversity.

generated by the loci in the minimal subset. VNTR 3336, which had the second highest allelic diversity, was repeatedly difficult to amplify. Therefore, this locus was also not included in the subset of 9 nonredundant loci and we do not recommend its routine use.

The above minimal subset was defined by analyzing all isolates and genotypes included in panel 3. We further analyzed the relative variability of the 29 MIRU-VNTR loci by identifying specifically those that varied within each of the four best identified lineages in this panel, as defined by the congruence of their MIRU-VNTR, spoligotyping, and IS*6110* RFLP profiles (corresponding to groups G, H, J, and K, see below). We

TABLE 4. Number of different patterns observed among the 68 *M. bovis* isolates in panel 3 and calculated genotypic diversities of different subsets of VNTRs

Combination of loci	No. of different patterns	Genotypic diversity
VNTR 3232, ETR-A and B	21	0.86
VNTR 3232, ETR-A and B, MIRU 26	24	0.87
VNTR 3232, ETR-A and B, MIRU 26, QUB11b	27	0.89
VNTR 3232, ETR-A and B, MIRU 26, QUB11b, QUB11a	28	0.90
VNTR 3232, ETR-A and B, MIRU 26, QUB11b, QUB11a, ETR-C, VNTR 4156, MIRU 4	32	0.91
MIRU-VNTR 29 loci	32	0.91

reasoned that loci that were variable within given lineages were more likely to have higher evolutionary rates because the isolates distinguished were mutually closely related. The 8 loci that varied in either of these lineages (Fig. 4) consistently comprised the 9 loci in the discriminatory subset defined in the whole of panel 3.

The simultaneous occurrence of two different alleles (9 and 10) was clearly detected for locus QUB 11a, included in the discriminatory subset, for one isolate obtained from an individual animal (96427). This observation indicates the presence of a clonal variant in the bacterial population from this isolate.

Genetic relationships (panel 3). The congruence of the genetic relationships, as defined by IS*6110* fingerprints, spoligotyping, and MIRU-VNTR markers, was analyzed for the isolates of panel 3 on the basis of the MIRU-VNTR genotypes (corresponding to the most discriminatory method) using the neighbor-joining distance algorithm (Fig. 4). The groupings defined by MIRU-VNTR typing were the most clearly congruent with those apparent from RFLP patterns and spoligotyping for the isolates of RFLP groups G, H, J, and K. For instance, group G was initially defined by similar RFLP patterns that varied from 8 to 11 IS*6110* elements. Samples of this group were distinguished by MIRU-VNTR into 5 different patterns but were grouped together based on variations involving at most a one-repeat difference in a single locus. The isolates of this group all had a single specific spoligotype, SB0162. The

consistency of RFLP group K, initially defined by a 4- to 6-band RFLP pattern, was likewise confirmed by mutual MIRU-VNTR variations involving at most 3 loci and by identification of a single, albeit nonspecific, spoligotype, SB0120. In contrast and not surprisingly, the isolates previously assigned to group A on the basis of their single-banded IS*6110* RFLP pattern appeared heterogeneous both by spoligotyping and by MIRU-VNTR typing (13 and 17 different patterns, respectively).

We quantified the congruence of the genetic relationships based on MIRU-VNTR typing on the one hand and the combination of IS*6110* RFLP and spoligotyping on the other to reduce biases due to the much lower resolution of the two latter methods when taken individually. When the whole of panel 3 was considered, these methods were found to correlate ($r = 0.30$) with a high degree of significance ($P < 10^{-4}$). Again, it was no surprise to find that when the single-banded IS*6110* fingerprints (group A) were excluded from the comparison, the correlation increased to $r = 0.63$ with an identical threshold of significance.

DISCUSSION

MIRU-VNTR typing is a useful tool for genotyping *M. tuberculosis* (2, 8, 15). Such a method has recently been shown to be more discriminatory than spoligotyping for *M. bovis* when using a limited number of isolates from Northern Ireland (25) or from Chad (14). However, the epidemiological relevance of this method has been difficult to appreciate so far, notably because of the lack of detailed epidemiological information about the isolates involved. Here, we have identified the most informative markers from a screen of 29 MIRU-VNTR loci by using a larger panel of isolates, representative of the genotypic diversity in a nationwide collection, together with cattle tracing information. Also in contrast to previous studies, a more stringent analysis of the resolution power and stability of the markers was possible by comparing them with both IS*6110* RFLP and spoligotyping, especially as half of our collection included *M. bovis* isolates with high IS*6110* copy numbers rarely reported elsewhere.

As a prerequisite to using them to trace transmission chains, genetic markers must be adequately stable to identify isolates of the same strain. As Belgium has an extremely low incidence of bovine tuberculosis (declared officially free of cattle tuberculosis in June 2003 according to the European Commission decision 2003/467/EC) and has no known environmental reservoir for *M. bovis*, it is likely that an outbreak on a farm is most often caused by a single source and thus involves a single strain or transmission chain. In our study, all but one series of samples from outbreak episodes covering up to 4 years on the same farm were perfectly matched by fully identical MIRU-VNTR alleles over the 29 target loci. The only exception was observed for two pooled samples, both displaying 2 alleles simultaneously in locus 3232. This observation could reflect the presence of two independent strains in these samples or the stochastic emergence of a clonal variant for this locus.

Furthermore, we also found that the MIRU-VNTR loci were stable in transmission chains covering at least 2 years and up to 5 different herds. Again, a single exception was observed for one isolate in one cluster, which had both a one-repeat difference in ETR-A and one IS*6110* band less in relation to the other isolates in this cluster. Interestingly, this isolate was obtained in 2000 from the farm presumably at the origin of this cluster (CE1) and where an outbreak was suspected to have started in 1996 or earlier. Therefore, this isolate could correspond to a glimpse into progressive clonal diversification over longer periods of time. In contrast, a couple of isolates with fully identical MIRU-VNTR types had slightly distinct multibanded IS*6110* RFLP patterns, although they were clearly epidemiologically linked. Such a phenomenon, which has also been observed for epi-linked *M. tuberculosis* isolates (1), suggests that IS*6110* RFLP may evolve very fast in certain *M. bovis* strains, and therefore, the use of IS*6110* fingerprinting alone might in some cases underestimate ongoing transmission of tuberculosis in cattle.

Among the isolates without known epidemiological links, MIRU-VNTR typing provided a higher discriminatory power than the two other methods, taken individually or in combination. As expected, most spoligotyping- or low-IS*6110*-copy-number-fingerprint-based clusters were distinguished by MIRU-VNTR. MIRU-VNTR typing was even able to distinguish a total of 7 isolates in IS*6110* RFLP fingerprint-based clusters with intermediate or high IS*6110* copy numbers. Similar observations have been recently obtained for epidemiologically unlinked *M. tuberculosis* isolates clustered by identical high-IS*6110*-copy-number fingerprints (17, 33). Conversely, none of the MIRU-VNTR clusters was split by spoligotyping. The epidemiological significance of three isolates in MIRU-VNTR clusters, distinguished by a single-band change in their intermediate- or high-IS*6110*-copy-number fingerprint, as well as other isolates with identical genotypes from the three methods, remains unknown.

The observed congruence between MIRU-VNTR, IS*6110* RFLP, and spoligotyping was found to be highly significant. This congruence is consistent with the clonal population structure of *M. bovis* (28) and indicates that the three independent markers are generally phylogenetically informative. Not surprisingly, however, IS*6110* RFLP appeared to be less reliable for identifying consistent genetic groups in cases of fingerprints with a single band. One lineage accounting for about half of all isolates collected in Belgium was clearly identified by the three methods. The corresponding spoligotype is quite specific, as it has only been reported so far in one French region (named as type F002 in reference 13). We verified that these isolates had the RD4 genomic deletion (data not shown) typical of classical *M. bovis* (4). In contrast, not a single representative of the SB0140 spoligotype, which is, according to www.mbovis.org, the most common spoligotype in the United Kingdom, was found in our collection. Thus, the *M. bovis* population differs greatly between the cattle from Belgium and Northern Ireland.

Our study collection includes more than 20% of the samples from bovine tuberculosis cases with positive culture collected over a 9-year period in Belgium and includes isolates representative of all the different spoligotypes and/or IS*6110* fingerprints available from a nationwide collection. The degree of epidemiological representation of this collection is difficult to ascertain, as information about potential links between the farms involved was not systematically available. This limitation also applies to the previous studies evaluating molecular typing methods of *M. bovis*. Despite these limitations, it is remarkable

that some loci systematically emerge among the most discriminatory markers across different settings. Our set of MIRU-VNTR loci included 25 loci in common with the set of 30 tested in Ireland (26) and incorporated the 16 loci recently tested on 67 isolates in a Chadian setting (14). In our case, the maximal resolution of MIRU-VNTR typing was already achieved using a subset of 9 loci (VNTR 3232, ETR-B, ETR-A, MIRU-26, QUB11b, QUB11a, ETR-C, VNTR 4156, and MIRU 4). Consistently, the differences among closely related isolates within well-identified clonal lineages were virtually all confined to these loci, indicating a generally higher evolutionary rate compared to the other loci. Moreover, about ninety percent of this resolution was concentrated in a core set of only 6 loci with comparatively high allelic diversities (VNTR 3232, ETR-A and B, MIRU 26, and QUB 11a and 11b). Interestingly, these 6 loci were all ranked among the markers with the highest allelic diversities in Ireland (25–27). In the Chadian study, high allelic diversities were also found for MIRU 26, ETR-A, and ETR-B, although a comparatively lower allelic diversity was observed for VNTR 3232, and neither QUB 11a nor 11b was tested. The three remaining loci (ETR-C, VNTR 4156, and MIRU 4) were marginally more polymorphic in our study, as in the Irish studies (25–27), but their contribution resided in the nonredundant discrimination of a few specific genotypes by single locus variations. Alternative, albeit larger, combinations of loci common to both studies were, however, possible to reach nearly maximal resolution. The core set (VNTR 3232, ETR-A and B, MIRU 26, and QUB 11a and 11b) of highly discriminatory loci, both in Ireland and Belgium, with radically distinct bacterial populations (see above), appear to be the most interesting candidates for testing the discrimination of *M. bovis* isolates on a more general scale. A future multicenter study should determine the performance of this core set for the general first-line discrimination of *M. bovis* isolates in different countries. The above comparisons suggest that secondary typing with a few additional loci potentially specific to some settings may provide additional limited discrimination of a few particular genotypes.

In conclusion, this study indicates that MIRU-VNTR markers provide a good balance between stability and variability compared to spoligotype and IS*6110* RFLP analysis and may be useful molecular tools for epidemiological studies of *M. bovis*. Their stability in virtually all epidemiologically related isolates analyzed so far support their use, at least as an exclusion method, i.e., even small differences in MIRU-VNTR genotypes can be interpreted as evidence of the absence of a link, with a high degree of confidence. In countries with a low incidence of disease, such as Belgium, the resolution may even be sufficient to identify different transmission chains. In addition, MIRU-VNTR typing is a convenient, rapid, and reproducible technique, especially compared to IS*6110* RFLP. Eventually, the portability of MIRU-VNTR data and the development of a standardized format integrated into an international database could be very useful for tracing the worldwide dissemination of the pathogen.

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