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Evaluation of Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat Analysis and Spoligotyping for Genotyping of *Mycobacterium bovis* Isolates and a Comparison with Restriction Fragment Length Polymorphism Typing[▽]

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Common strain typing methods for differentiation of *Mycobacterium bovis* isolates include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, spoligotyping, and, more recently, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing. MIRU-VNTR typing and spoligotyping were evaluated in this study, and these typing methods were compared with RFLP typing. A total of 386 *M. bovis* isolates from cattle, badgers, and deer in the Republic of Ireland that had previously been typed by IS*6110*, polymorphic GC-rich sequence (PGRS), and direct-repeat (DR) RFLP were included in the study. Spoligotyping and analysis of six VNTR loci (QUB 11a, QUB 11b, ETR A, 4052, MIRU 26, and 1895) were performed on the samples. RFLP analysis was the method that gave the greatest differentiation of strains, with a Hunter-Gaston discriminatory index (HGDI) of 0.927; the HGDI recorded for MIRU-VNTR typing was marginally lower at 0.918, and spoligotyping was the least discriminatory method, with an HGDI of 0.7. Spoligotype SB0140 represented approximately 50% of the isolates. Within the group of isolates represented by SB0140, there was a much lower level of concordance between RFLP and MIRU-VNTR typing than for groups represented by other spoligotypes. A combination of spoligotyping and MIRU-VNTR typing offered advantages over MIRU-VNTR typing alone. In a combined spoligotyping and MIRU-VNTR typing protocol, the number of VNTR loci could be reduced to four (QUB 11a, QUB 11b, ETR A, and 4052) while maintaining a high level of strain differentiation.

The development of molecular techniques for differentiation of *Mycobacterium bovis* isolates has been of considerable benefit in epidemiological studies. Typing methods that have been commonly used include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, spoligotyping, and, more recently, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing (7, 19).

RFLP analysis of *M. bovis* isolates has commonly utilized polymorphism of the insertion sequence IS*6110* and repetitive DNA elements such as the polymorphic GC-rich sequence (PGRS) and the direct-repeat (DR) region. Analysis of polymorphism of IS*6110*, the PGRS, and the DR region in combination has provided a high level of discrimination between strains (7, 19). REA has been widely used in New Zealand and has also given excellent resolution of strains (4). However, both RFLP analysis and REA require relatively large quantities of DNA and are laborious and time-consuming procedures. Complex banding patterns make analysis and interlaboratory comparisons difficult. Spoligotyping is a PCR-based typing method that reveals the presence or absence of unique spacer sequences located between the direct-repeat sequences of the DR region (12). It is a relatively easy procedure to perform, and the results can be expressed in a digital format. However, spoligotyping does not differentiate *M. bovis* strains to the same extent as RFLP analysis or REA (7, 19). Minisatellite-like loci in the *Mycobacterium tuberculosis* complex genome, described as mycobacterial interspersed repetitive units, may show polymorphism of the number of tandem repeats. A wide range of *M. tuberculosis* complex MIRU-VNTR loci have been evaluated, and loci which are informative for *M. bovis* isolates have been identified (8, 16, 17, 20, 23). Similar to spoligotyping, MIRU-VNTR typing has the advantages of ease of procedure and the generation of results in a digital format.

In recent years, genotyping by IS*6110*, PGRS, and DR RFLP has been used in epidemiological studies of *M. bovis* infection in the Republic of Ireland (5, 6, 14). While RFLP analysis has given a high level of strain differentiation, its replacement by MIRU-VNTR typing or by a combination of MIRU-VNTR typing and spoligotyping offers potential advantages. The objective of this study was to evaluate MIRU-VNTR typing or a combination of MIRU-VNTR typing and spoligotyping for discrimination of *M. bovis* strains, to compare the discriminatory powers of the two methods against RFLP analysis, and to investigate the level of concordance between the three typing systems.

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TABLE 1. Primer sequences for MIRU-VNTR typing

VNTR locus	Alternate name	Primer pair with label (5′ → 3′)[a]	Reference
QUB 11a	2163a	Hex-CCCATCCCGCTTAGCACATTCGTA, TTCAGGGGGGATCCGGGA	20
QUB 11b	2163b	Hex-CGTAAGGGGGATGCGGGAAATAGG, CGAAGTGAATGGTGGCAT	20
1895		Fam-GGTGCACGGCCTCGGCTCC, AAGCCCCGCCGCCAATCAA	16
2165	ETR A	Fam-AAATCGGTCCCATCACCTTCTTAT, CGAAGCCTGGGGTGCCCGCGATTT	8
2996	MIRU 26	Hex-TAGGTCTACCGTCGAAATCTGTGAC, CATAGGCGACCAGGCGAATAG	23
4052	QUB 26	Hex-AACGCTCAGCTGTCGGAT, GGCCAGGTCCTTCCCGAT	20

[a] The forward primer of the primer pair was labeled with a fluorescent dye to facilitate with the detection of the amplified product. Hex, hexchlorofluorescein; FAM, 6-carboxyfluorescein.

MATERIALS AND METHODS

Mycobacterial strains and culture procedure. Stored *M. bovis* isolates that had previously been typed by RFLP analysis (5, 6) were used in this study. Isolates that had been stored at −20°C were thawed and cultured in 3 ml of Middlebrook 7H9 broth at 37°C for 7 days. Aliquots (0.5 ml) of the Middlebrook 7H9 broth were streaked onto Stonebrinks medium and Lowenstein-Jensen medium containing pyruvate (prepared as solid slants in screw-cap tubes), incubated at 37°C, and monitored on a weekly basis. Cultures suitable for DNA extraction were obtained for 386 isolates. The isolates had been obtained from 243 badgers, 119 cattle, and 24 deer during the years 1996 to 2002. The isolates were obtained from all areas of the Republic of Ireland; however, a total of 206 originated in four study areas described by Griffin et al. (9).

DNA extraction. Colonies were transferred from the slopes into microtubes containing 500 μl of phosphate-buffered saline with Tween 20 (PBS-Tw) (Sigma Aldrich, Wicklow, Ireland). The microtubes were placed in a heating block at 100°C for 15 min to heat lyse the cells and vortexed periodically. Microtubes were centrifuged at 6,000 × *g* for 2 min. The supernatant was transferred into a clean, labeled 1.5-ml Eppendorf tube. DNA template was stored at −20°C.

VNTR typing. VNTR typing was performed using the six loci QUB 11a, QUB 11b, ETR A, MIRU 26, 4052, and 1895. The six genomic loci were amplified in separate PCRs with the primers described in Table 1. Reaction volumes of 25 μl containing 2.5 μl of 10× PCR buffer (Qiagen, West Sussex, United Kingdom), 0.2 μl of 50 pmol primer set, 2 μl (100 μM) of each of four deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), 5 μl of Q solution, 0.125 μl of Hotstar *Taq* polymerase (1 unit) (Qiagen), and 9.175 μl of pure H_2O. Template DNA (5 μl) was added to each PCR mix. A DNA extract from *M. bovis* and *M. tuberculosis* H37 was included in each set of reactions as a positive control and sterile distilled water as a negative nontemplate control. Amplification was performed in a Flexigene thermocycler with an initial activation step of 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min. The final extension was 72°C for 10 min. When the PCR was complete, the amplified products were stored light protected at −18°C until ready to be run on the MegaBACE 1000 (GE Healthcare Life Sciences, United Kingdom). The forward primer of the primer pair was labeled with a fluorescent dye (Table 1) to facilitate the detection of the amplified product. PCR products were diluted 1:50 in molecular-grade water and separated on a 96-capillary MegaBACE 1000 sequencer using Rox-labeled MegaBACE ET900-R as a size standard. The electrophoresis was run for 120 min using MegaBACE matrix, with an injection voltage of 3 kV for 45 s and a running voltage of 10 kV. Each peak was identified according to color and size and assigned to a distinct allele number.

Spoligotyping. Spoligotyping was performed according to the method described by Kamerbeek et al. (12) except that a digoxigenin labeling and detection system (Roche Diagnostics, West Sussex, United Kingdom) was used. Spoligotype patterns were given the names assigned in the *M. bovis* spoligotyping database at http://www.mbovis.org.

Statistical analysis. Calculation of the discriminatory power of each typing method was based on Simpson's index of diversity as described by Hunter and Gaston (11). This value is commonly referred to as the Hunter-Gaston discriminatory index (HGDI). Wallace's coefficient was used to quantify the level of concordance between typing methods (3). This calculates the degree to which one typing method can predict the result of another typing method. A high value of Wallace's coefficient suggests that the use of both methods is redundant. Wallace's coefficient was calculated using the Web tool http://www.comparingpartitions.info. The allelic diversity at the different VNTR loci was calculated using the method described by Selander et al. (18).

RESULTS

Resolution of strains. RFLP analysis, with an HGDI of 0.927 was more discriminating than MIRU-VNTR typing, which had a HGDI of 0.918, while spoligotyping was the least discriminatory of the three methods (Table 2). There were 65 RFLP profiles, which were divided into 33 clusters and 32 unique isolates, compared to 41 VNTR profiles comprising 26 clusters and 15 unique isolates. The largest RFLP cluster contained 58 isolates, while the largest MIRU-VNTR cluster contained 65

TABLE 2. Comparison of discriminatory powers of various genotyping protocols

Procedure	HGDI[a]	No. of:			
		Profiles	Clusters	Unique isolates	Isolates in largest group
RFLP analysis	0.927	65	33	32	58
VNTR typing	0.918	41	26	15	65
Spoligotyping	0.700	15	14	1	194
Spoligotyping + RFLP analysis	0.929	68	35	33	58
Spoligotyping + VNTR typing with:					
6 loci	0.933	54	36	18	61
5 loci[b]	0.930	51	34	17	63
4 loci[c]	0.930	49	34	15	63
Spoligotyping + RFLP analysis + VNTR typing	0.958	104	51	53	55

[a] HGDI, Hunter-Gaston discriminatory index.
[b] VNTR loci QUB 11a, QUB 11b, ETR A, 4052, and 1895.
[c] VNTR loci QUB 11a, QUB 11b, ETR A, and 4052.

TABLE 3. Resolution of eight VNTR clusters by spoligotyping

VNTR profile[a]	Spoligotype	No. of isolates
10 3 5 5 4 3	SB0140	20
	SB0993	3
10 4 6 5 4 4	SB0140	19
	SB0273	10
11 2 6 5 4 4	SB0140	2
	SB0269	1
11 3 7 5 4 4	SB0140	6
	SB0144	12
	SB0486	2
11 4 5 5 4 4	SB0140	2
	SB0054	6
11 4 7 5 3 4	SB0140	14
	SB0141	7
	SB0486	5
	SB0145	5
11 4 7 6 4 3	SB0140	1
	SB0142	46
	SB0995	2
11 3 7 3 3 2	SB0120	61
	SB0146	3
	SB0998	1

[a] The VNTR loci are listed in the order QUB 11a, QUB 11b, ETR A, MIRU 26, 4052, and 1895.

isolates. Spoligotyping identified 14 clusters and one unique isolate; the largest cluster, represented by spoligotype pattern SB0140, contained approximately 50% of the isolates. Spoligotyping produced further resolution of eight MIRU-VNTR clusters (Table 3). RFLP clusters were not resolved to the same extent by spoligotyping, with only three being further subdivided. The allelic diversity of the VNTR loci ranged from 0.44 for VNTR QUB 11a to 0.57 for VNTR 1895. (Table 4).

Typing system concordance. The level of concordance between the typing systems varied according to spoligotype. Wallace's coefficient (3), which is a measure of the degree to which one typing method can predict the result of another typing method, was used to quantify the level of concordance between typing methods (Table 5). Strains bearing spoligotype SB0140 showed highly variable RFLP and VNTR profiles and a low level of concordance between these two typing methods. The highest level of concordance was found in the strains that did not have the SB0140 spoligotype pattern.

TABLE 4. Allelic diversity of VNTR loci

Locus	Allelic diversity	No. of isolates with VNTR allele:										
		1	2	3	4	5	6	7	8	9	10	11
QUB 11a	0.44			7			8			13	82	276
QUB 11b	0.48	5	5	130	245	1						
ETR A	0.45		1	4	1	48	54	277		1		
MIRU 26	0.49		1	68	2	260	55					
4052	0.55		45	110	231							
1895	0.57		70	89	227							

TABLE 5. Potential of one typing system (reference typing system) to predict the outcome of an alternative typing system (secondary typing system) as measured by the Wallace coefficient

Reference typing system	Secondary typing system	Wallace coefficient for group[a]:		
		1 (n = 386)	2 (n = 194)	3 (n = 192)
RFLP analysis	VNTR typing	0.56	0.25	0.89
VNTR typing	RFLP analysis	0.49	0.35	0.66
Spoligotyping	RFLP analysis	0.24	0.14	0.74
	VNTR typing	0.22	0.10	0.87

[a] Group 1, all isolates; group 2, isolates with spoligotype pattern SB0140; group 3, isolates that did not have spoligotype pattern SB0140.

There was a close correlation between MIRU 26 alleles and spoligotype. A five-repeat allele at the MIRU 26 locus was characteristic of 187 of the 194 isolates represented by spoligotype SB0140, a three-repeat allele was present in 62 of the 64 isolates represented by spoligotype SB0130, and a six-repeat allele was present in all of the 49 isolates represented by spoligotype SB0142. At the VNTR 1895 locus, a two-repeat allele was present in all of the spoligotype SB0130 isolates. Consequently, when a combined spoligotyping and MIRU-VNTR typing protocol was used, the omission of MIRU 26 and 1895 resulted in only a slight reduction of strain resolution (Table 2).

Geographic and species distribution. Spoligotype SB0140 was widely distributed throughout Ireland. Nine VNTR types represented 71% of the SB0140 isolates and were also widely distributed geographically. Another 23% of isolates represented by spoligotype SB0140 were subdivided by MIRU-VNTR typing into geographically localized clusters. Sixty-four isolates were represented by spoligotype SB0130 and were widely distributed throughout the south of the country. In contrast to the case for spoligotype SB0140, there was little diversity of VNTR types within the spoligotype SB0130 cluster, with 95% of the isolates represented by a single VNTR profile. The third most frequent spoligotype was SB0142. This was found predominantly in three counties in the northeast. Like for spoligotype SB0130, there was little diversity of VNTR types, with 94% of the isolates represented by a single VNTR type. Isolates represented by spoligotype SB0273 were found in two widely separated counties (Donegal and Kilkenny). However, differences in RFLP and VNTR profiles suggested that these were two phylogenetically unrelated groups. All of the prevalent VNTR profiles were shared by strains from cattle, badgers, and deer. This is consistent with previous findings that spoligotypes and RFLP types were shared by strains from all three species (5).

DISCUSSION

In this study, MIRU-VNTR typing using a panel of six loci was an easy-to-apply and reliable technique that provided good differentiation of strains. The six VNTR loci were selected based on an initial evaluation of a panel of 24 loci in 60 *M. bovis* isolates (unpublished data). The allelic diversities recorded for loci QUB 11b, 2165, MIRU 26, and 4052 were very similar to findings in other studies in Northern Ireland (17), Italy (2), Spain (15), and the United States (13). The allelic

diversity of VNTR locus QUB 11a was found to be low in studies in Spain (15) and the United States (13) but was satisfactory in the present study, as was the case in Northern Ireland (17). In this study VNTR 1895 had the highest allelic diversity of the six loci, in contrast to previous studies (2, 13, 17). There are other VNTR loci that have proved useful for discrimination of *M. bovis* strains that were not evaluated in this study. ETR B (VNTR 2461) produced good resolution of *M. bovis* strains in a number of studies (2, 10, 13, 21). In a study of seven VNTR loci in Northern Ireland, VNTR 3232 produced the greatest resolution of *M. bovis* stains (21). However, difficulties with the reproducibility of typing VNTR 3232 have been reported (2, 13).

There is little information available on the discriminatory power of MIRU-VNTR typing compared to RFLP analysis. Allix et al. (1) found that in a panel of 68 *M. bovis* isolates, a combination of three VNTR loci (3232, ETR A, and ETR B) had a genotypic diversity of 0.86, compared to 0.73 for IS*6110* RFLP. In the present study, RFLP analysis using three probes (IS*6110*, the PGRS, and the DR region) produced 65 different profiles, while MIRU-VNTR typing of this panel of isolates gave 41 different profiles. However, almost 50% of the RFLP profiles were unique to one isolate, and the discriminatory powers of the two methods as measured by the Hunter-Gaston discriminatory index were comparable (Table 2).

A combination of spoligotyping and MIRU-VNTR typing offers some advantages over MIRU-VNTR typing alone. A few VNTR profiles were common to more than one spoligotype (Table 3) and were identified in isolates from diverse geographic regions. Isolates bearing these VNTR types could usually be subdivided into geographically localized clusters by spoligotyping. In addition, spoligotyping may provide useful phylogenetic information (22). Some alleles of the MIRU 26 and 1895 loci had a linkage disequilibrium with spoligotyping. Consequently, these two loci were to a large extent redundant in a combined spoligotyping and VNTR protocol, and only the four VNTR loci QUB 11a, QUB 11b, ETR A, and 4052 were required.

SB0140 is the spoligotype most frequently identified in *M. bovis* isolates in Ireland and Great Britain. This spoligotype has previously been referred to as type A1 (5) and VLA type 9 (22). There was a high level of diversity of RFLP and VNTR profiles within the group of 194 isolates represented by SB0140 and a lower degree of concordance between VNTR and RFLP types than found within groups of isolates represented by other spoligotypes. In agreement with our findings, high levels of both genetic and phenotypic diversity were found among strains bearing SB0140 in Great Britain (24). The most common VNTR types within the SB0140 group were widely distributed geographically, which limited their usefulness for tracing the geographic spread of infection. In contrast, most of the less common VNTR types within the SB0140 group were largely concentrated in defined geographic areas.

With few exceptions, the other spoligotypes were also concentrated in defined geographic areas. The most geographically dispersed was SB0130, which was distributed over several counties in the south. This was the second most common spoligotype identified, and interestingly, it does not belong to the SB0140 clonal complex described by Smith et al. (22). This clonal complex is identified by the deletion of spacers 6 and 8 to 12, and it includes the majority of *M. bovis* strains in Ireland and Great Britain. However, in contrast to the case for SB0140, there was very little diversity of VNTR profiles within the SB0130 group, which suggests that it has undergone a more recent clonal expansion in Ireland than SB0140.

The optimal procedure to use for strain typing of *M. bovis* will depend on the strains present in a region, the number of isolates to be typed, the resources available, and the degree of resolution required. A combination of spoligotyping and typing of four VNTR loci offers a relatively uncomplicated procedure suitable for high-throughput typing. This study has shown that a protocol using VNTR loci QUB 11a, QUB 11b, ETR A, and 4052 combined with spoligotyping gave a level of discrimination of *M. bovis* strains that was comparable to that produced by IS*6110*, PGRS, and DR RFLP.

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