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An evaluation of MIRU-VNTR analysis and spoligotyping for genotyping of M. bovis isolates and a comparison with RFLP typing.

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Comment [D1]: Joanne, I think you should put yourself down as both Backweston and DIT. This will be useful when it comes to the M.Phil. Also, you may not be aware of the fact that the last author is quite an important position and I am not sure if I deserve that position. I am happy to stay there is you wish but if you think someone else deserves it more than fell free to move me.

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. Strain typing of *Mycobacterium bovis* isolates based on the variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) and on spoligotyping was evaluated in this study and these typing methods were compared with restriction fragment length polymorphism (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 IS6110, polymorphic GC-rich sequence (PGRS) and direct repeat (DR) RFLP were included in the study. Spoligotyping and analysis of six VNTR loci (2163a, 2163b, 2165, 4052, 2996 and 1895) was performed on the samples. RFLP 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 was marginally lower at 0.918 and spoligotyping was the least discriminatory method with a 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 compared to 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 (2163a, 2163b 2165 and 4052) while maintaining a high level of strain differentiation.

Comment [D2]: Not sure what the word limits for the abstract are but it might be useful to include the sentence before your start if possible.

INTRODUCTION

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 IS6110, and repetitive DNA elements such as the polymorphic GC-rich sequence (PGRS) and the direct repeat (DR) region. Analysis of polymorphism of IS6110, PGRS and DR 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 and REA require relatively large quantities of DNA and are laborious and time-consuming procedures. Complex banding patterns makes analysis and inter-laboratory 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 or REA (7, 19). Mini-satellite-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 has the advantages of ease of procedure and the generation of results in a digital format.

In recent years genotyping by IS6110, 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 and spoligotyping for discrimination of *M. bovis* strains, to compare the discriminatory power of both methods against RFLP analysis and to investigate the level of concordance between the three typing systems.

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^{o}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 (PBS) with Tween 20 (PBS-Tw) and were heat killed at 80 °C for 1 hour in a pre-heated water bath. The cells were washed twice with PBS-Tw. The supernatant was discarded and aliquots (500 µl) of pure sterile water were added to the pellet and the cells were re-suspended. 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 6000g for 2 min. The supernatant was transferred into a clean, labelled 1.5 ml Eppendorf tube. DNA template was stored at -20°C.

VNTR typing. VNTR typing was performed using the six loci 2163a, 2163b, 2165, 2996, 4052 and 1895. The 6 genomic loci were amplified in separate PCR reactions with primers described in Table 1. Reaction volumes of 25 µl containing 2.5 µl of 10X PCR buffer (Qiagen), 0.2 µl of 50 pmol primer set, 2 µl (100 µM) of each of the four deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP, 5 µl of Q solution, 0.125 µl of Hotstar Taq (1 unit) and 10.175 µl of pure H_20. Template DNA (5 µl) was added to each PCR reaction mix. A DNA extract from *M . bovis* H37 was included in each set of reactions as a positive control and sterile distilled water as a negative non-template 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 sec, 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 run on the MegaBACE 1000. The forward primer of the primer pair was labelled with a Fluorescent dye (Table 1), to facilitate the detection of the amplified product using the MegaBACE 1000 Instrument. PCR products were diluted 1:50 in molecular-grade water and separated on a 96-capillary MegaBACE ^{TM} 1000 Sequencer (GE Healthcare Life Sciences) using Rox-labelled 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 10kV. Each peak was identified according to colour and size and assigned to a distinct allele number.

Comment [D3]: Normally labels are on the 5' end of primers and not the 3'end. Just check if this is the case and fix the table if necessary.

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

Comment [EC4]: You will need to describe the adjustment

Statistical analysis. Calculation of the discriminatory power of each typing method was based on Simpsons 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 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 with a HGDI value of 0.927 was more discriminating than MIRU-VNTR, which had a HGDI value of 0.918, while spoligotyping was the least discriminatory of the three methods (Table 2). There were 65 RFLP profiles that 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 isolates. Spoligotyping identified 14 clusters and 1 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 2163a 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 SB0140 spoligotype pattern

There was a close correlation between VNTR 2996 alleles and spoligotype. A five repeat allele at the VNTR 2996 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 VNTR 2996 and 1895 resulted in only a slight reduction in strain resolution (Table 2).

Geographic and species distribution. Spoligotype SB0140 was widely distributed throughout the country. 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 localised clusters. Sixty four isolates were represented by spoligotype SB0130 and were widely distributed throughout the south of the country. In contrast to 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 North East. Like 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. bo*vis isolates (unpublished). The allelic diversities recorded for loci 2163b, 2165, 2996 and 4052 was very similar to findings in other studies in Northern Ireland (17),

Italy (2), Spain (15) and the USA (13). The allelic diversity of VNTR locus 2163a was found to be low in studies in Spain (15) and the USA (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. VNTR 2461 (ETR B) produced good resolution of *M. bovis* strains in a number of studies (2, 10, 13, 21). In a study of 7 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. Allix et al (1) found that in a panel of 68 *M. bovis* isolates a combination of three VNTR loci (3232, 2165 and 2461) had a genotypic diversity of 0.86 compared to 0.73 for IS6110 RFLP. In the present study RFLP analysis using three probes (IS6110, PGRS and DR) 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 power of both methods as measured by the Hunter-Gaston discriminatory index was 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 localised clusters by spoligotyping. In addition, spoligotyping may provide useful phylogenetic information (22). Some alleles of VNTR loci 2996 and 1895 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 2163a, 2163b 2165 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 compared to that found within groups of isolates represented by other spoligotypes. In agreement with our findings a high level of both genetic and phenotypic diversity was found amongst strains bearing SB0140 in Great Britain (24). The most common VNTR types within the SB0140 group were widely distributed geographically. These may represent ancestral VNTR profiles associated with SB0140 strains in Ireland. The widespread distribution of these VNTR types limits their utility in tracing 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 accounts for the majority of M. bovis strains in Ireland and Great Britain. However, in contrast to 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 compared to 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 this protocol gave a level of strain discrimination that was comparable to that produced by combined IS6110, PGRS and DR RFLP typing.

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TABLE 1. Primer Sequences for MIRU-VNTR Typing

MIRU-VNTR Locus	Alias	Primer Pair with Label (5'-3')[a]	Reference
2163a	QUB 11a	CCCATCCCGCTTAGCACATTCGTA Hex TTCAGGGGGGATCCGGGA	20
2163b	QUB 11b	CGTAAGGGGGATGCGGGAAATAGG Hex CGAAGTGAATGGTGGCAT	20
1895	QUB 1895	GGTGCACGGCCTCGGCTCC Fam AAGCCCCGCCGCCAATCAA	16
2165	ETR-A	AAATCGGTCCCATCACCTTCTTAT Fam CGAAGCCTGGGGTGCCCGCGATTT	8
2996	MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC Hex CATAGGCGACCAGGCGAATAG	23
4052	QUB 26	AACGCTCAGCTGTCGGAT Hex GGCCAGGTCCTTCCCGAT	20

[a] The forward primer of the primer pair was labelled with a Fluorescent dye to facilitate with the detection of the amplified product.

Comment [D5]: Look at the position of the labels.

TABLE 2. Comparison of the discriminatory power of various genotyping protocols

Procedure	HGDI[a]	Profiles	Clusters	Unique isolates	Largest group
Spoligotyping	0.700	15	14	1	194
RFLP	0.927	65	33	32	58
VNTR	0.918	41	26	15	65
Spoligotyping + RFLP	0.929	68	35	33	58
Spoligotyping + VNTR (6 loci)	0.933	54	36	18	61
Spoligotyping + VNTR (5 loci)[b]	0.930	51	34	17	63
Spololigotyping + VNTR (4 loci)[c]	0.930	49	34	15	63
Spololigotyping + RFLP + VNTR	0.958	104	51	53	55

[a]Hunter Gaston Discriminatory Index.
[b]VNTR loci 2163a, 2163b, 2165, 4052 and 1895
[c]VNTR loci 2163a, 2163b, 2165 and 4052

Comment [D6]: I was wondering why you put spoligotyping first and you are primarily trying to compare the spoligityping and VNTR to the RFLP. I would be inclined to put RFLP first.

TABLE 3. Resolution of eight VNTR clusters by spoligotyping

VNTR profile[a]	Spoligotype	No 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 2163a, 2163b, 2165, 2996, 4052 and 1895.

TABLE 4. Allelic diversity of VNTR loci

Locus	Allelic diversity	Number of isolates with VNTR allele										
		1	2	3	4	5	6	7	8	9	10	11
2163a	0.44			7			8			13	82	276
2163b	0.48	5	5	130	245	1						
2 165	0.45		1	4	1	48	54	277		1		
2996	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		
		Group 1 (n = 386)	Group 2 (n = 194)	Group 3 (n = 192)
RFLP	VNTR	.56	.25	.89
VNTR	RFLP	.49	.35	.66
Spoligotyping	RFLP	.24	.14	.74
Spoligotyping	VNTR	.22	.10	.87

Group 1 = all isolates,
Group 2 = isolates with spoligotype pattern SB0140
Group 3 = isolates that did not have spoligotype pattern SB0140

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