Improved differentiation of *Mycobacterium tuberculosis* isolates in the north of England using additional variable number tandem repeat loci

N. GAUKRODGER, D. THOMPSON, S. J. SARGINSON, J. G. MAGEE and A. D. SAILS

Health Protection Agency, Institute of Pathology, Newcastle General Hospital, Newcastle upon Tyne NE4 6BE, UK

Accepted: 24 September 2010

ABSTRACT

Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) genotyping of over 3300 *Mycobacterium tuberculosis* isolates from the north of England has identified large clusters of strains which share common profiles. However, many apparent clusters identified when typed using the existing 15 loci lack clear epidemiological links. This study seeks to discover whether or not six additional VNTR loci can increase the discriminatory power of the existing MIRU-VNTR 15-loci technique. Two hundred and six *M. tuberculosis* isolates were genotyped, including 57 isolates from 20 epidemiologically linked clusters and 149 from unlinked patients belonging to six large MIRU-VNTR-defined clusters. The discriminatory power of the six additional loci was high (Hunter Gaston Discriminatory Index [HGDI]: 0.952). Five of the six loci were highly discriminative ($h>0.6$); however, locus 2401 was less discriminative ($h=0.5$). The additional VNTR loci were able to subtype all six unlinked common MIRU-VNTR clusters into 56 subclusters, significantly differentiating unrelated strains in a set previously incorrectly clustered using 15 MIRU-VNTR loci. The largest cluster size was 14 (9.3%) when typed using the six additional VNTR loci, compared to 30 (20%) when typed using the original 15 MIRU-VNTR loci. The same loci were also found to be stable as a result of their inability to subdivide any of the epidemiologically linked clusters. This study has demonstrated that expanding the MIRU-VNTR panel beyond the 15 previously used loci significantly increases the discriminatory power of the technique and thus provides a valuable tool in the epidemiological monitoring of this disease.

KEY WORDS: Bacterial typing techniques.
Mycobacterium tuberculosis.
VNTR loci.

Introduction

Tuberculosis (TB) remains a global threat to human health.[1] Recent developments in molecular typing techniques provide an opportunity to study the geographic and evolutionary relationships of strains of *Mycobacterium tuberculosis*. Polymerase chain reaction (PCR)-based typing of *M. tuberculosis* has already helped to establish a central database linking genotypes and epidemiological data, thus permitting better understanding of factors that influence TB transmission and the evaluation of regional control programmes.[2,3]

Typing based on the variable number of tandem repeats (VNTRs) of genetic elements named mycobacterial interspersed repetitive units (MIRUs) in *M. tuberculosis* is an established PCR-based technique.[4] A standard 12 MIRU-VNTR loci technique has been used in the USA[5,6] and Europe.[4,7–9] Further to this, an alternative set of 15 MIRU-VNTR loci (including 12 MIRU loci: 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40, plus three exact tandem repeat [ETR] loci: A, B, and C) is commonly used in the UK for routine epidemiological discrimination of *M. tuberculosis* isolates.[10–12] Application of the 15 MIRU-VNTR loci typing technique to over 3300 isolates has identified several large clusters of *M. tuberculosis* strains possessing profiles which are indistinguishable; however, such clusters have no known epidemiological links. Therefore, the technique may be insufficiently discriminatory for the identification of epidemiologically significant clusters of *M. tuberculosis* infections in the north of England.

The aim of this study is to investigate the possibility that additional VNTR loci could increase the discriminatory power of the existing 15 loci technique for epidemiological typing of *M. tuberculosis*.

Correspondence to: Nicole Gaukrodger
Pathology Department, Queen Elizabeth Hospital, Gateshead, Tyne and Wear NE9 6SX, UK
Email: nicole.gaukrodger@ghnt.nhs.uk

Materials and methods

Bacterial isolates and DNA extraction

A total of 207 *M. tuberculosis* isolates collected at the Health Protection Agency (HPA) North East Regional Laboratory between 2000 and 2007 were investigated. All the isolates had previously been investigated using the 15 MIRU-VNTR loci technique. The geographic origin of the strains spanned three regions in the north of England (North East, North West and Yorkshire & Humber). The first set comprised 57 isolates from 20 clusters with known epidemiological

links. The second set comprised 150 apparently unrelated isolates collected between 2002 and 2007. Previous MIRU-VNTR typing had grouped this set into six clusters representing the six most common profiles recognised in the North East Regional Laboratory database. Mycobacterial genomic DNA was prepared according to the method of van Embden *et al*.,[13] using heat lysis followed by centrifugation, with the extracted DNA being stored at –20˚C prior to use.

Design of PCR primers

To enable the use of non-denaturing high-performance liquid chromatography (non-dHPLC) for the sizing of PCR products, the primers were designed to produce PCR products of between 100 and 600 bp. Several of the VNTR loci primers from previously published studies have been demonstrated to produce amplification products of >600 bp, and therefore were unsuitable for analysis in the non-dHPLC instrument. A minimum of three target sequences from different *M. tuberculosis* strains (H37Rv, CDC1551 and F11) were downloaded from GenBank (National Center for Biotechnology Information [NCBI])[14] and a sequence alignment was performed using the MegAlign software application (Lasergene 7, DNAstar, USA)[15] to locate further conserved sequences either upstream or downstream from the published VNTR sequence. Specific oligonucleotide primers (Table 1) were designed to amplify VNTR loci 0424, 1955, 1982, 2163b and 2401 using the newly aligned consensus sequence.

Primers previously described by van Deutekom *et al*.[16] were demonstrated to produce amplification products of <600 bp and were therefore used to amplify VNTR locus 3690. Each primer was analysed in the PrimerSelect software application (Lasergene 7)[15] which automatically calculates the primer melting temperature (T_m) and predicts secondary structures such as primer dimers. None of the primer sequences had significant secondary structures. Potential primers were analysed for similarity to non-target sequences by performing a nucleotide-nucleotide BLAST search.[17] The primers in Table 1 showed no significant homology with any other non-target species. Primers were synthesised by Eurogentec, Belgium.

PCR amplification of MIRU-VNTR loci

Following optimisation of magnesium chloride concentration, primer concentration and primer annealing temperature, the PCR mixture was prepared in a 50 µL volume containing: 12 ng mycobacterial DNA, 4% dimethyl sulphoxide (Sigma, UK), 1.25 units AmpliTaq Gold DNA polymerase, 0.5 mmol/L 10 mmol dNTP mix and varying final concentrations of $MgCl_2$ and primers (Table 1) in 1x PCR buffer (Applied Biosystems, UK). The PCR reaction was carried out in an MBS Satellite thermal cycler (Thermo Electron Corporation, UK) under the following conditions: initial denaturation at 95˚C for 15 min, and then 35 cycles of 94˚C for 30 sec, optimum T_a for 30 sec (Table 1) and 72˚C for 1 min, followed by a final extension at 72˚C for 10 min. Positive control DNA (*M. tuberculosis* strain H37Rv) of known amplicon size and VNTR copy number and a negative control of mastermix excluding template DNA were incorporated in each PCR and detection assay.

Comparison of gel electrophoresis and non-dHPLC for sizing PCR products generated from the VNTR loci

The sizes of amplified DNA fragments were determined using a WAVE dHPLC instrument (WAVE Model L-7250, Transgenomic, UK) under non-denaturing conditions, as previously described by Evans *et al*.[18] The respective number of VNTRs per locus was then calculated for each isolate using the flanking region and VNTR unit size. The numbers of VNTRs were scored within a strict repeat integer range of ±0.25 repeats to ensure size accuracy on the WAVE instrument.[18] One hundred of the PCR products analysed on the WAVE dHPLC instrument were also analysed using conventional agarose gel electrophoresis to compare sizes and determine the reproducibility of the WAVE method. The sizes of the PCR products were determined by comparing the PCR product migration in the gel against 50- and 100-bp molecular weight DNA markers (Roche, UK).

Statistical analysis

The allelic diversity index (ADI) of each of the six additional VNTR loci was calculated using the equation $h=1–\Sigma\chi_i^2\ (n/[n–1])$, where χ_i is the frequency of the ith allele

Table 1. Polymerase chain reaction primer sequences and reaction conditions.

VNTR locus	PCR primers (5´ to 3´)	Primer length (bp)	$MgCl_2$ (mmol/L)	Primer pair (µmol/L)	T_a (˚C)
0424	F: CCGCCCTGGTCGTCTGGA	18	2.0	0.3	57.0
	R: CGACTCTCCGGCATCCTCAACAAC	24			
1955	F: GTCGTCGGTGCGGGCCACCAG	21	2.0	0.4	69.8
	R: CGGCCGTCTGTTCCGACGCCAATA	24			
1982	F: CGGAATGGCTACGGAAGGAATACT	24	1.5	0.5	62.0
	R: CCTTCAGTCTGCCGGCAATAACG	23			
2163b	F: CGTAAGGGGGATGCGGGAAATAGG	24	2.0	0.4	67.6
	R: CGAAGTGAATGGTGGCAT	18			
2401	F: TGGATTTGCTTACGTGGGCTGATT	24	1.0	0.3	57.0
	R: TCAAGCTAGCGCGTCTGCCATTCC	24			
3690*	F: CGGTGGAGGCGATGAACGTCTTC	23	1.0	0.3	65.0
	R: TAGAGCGGCACGGGGGAAAGCTTAG	25			

*Previously described primers by van Deutekom *et al*.[16]

Table 2. Application of additional VNTR loci to epidemiologically linked isolates.

Cluster	Incident type	No. isolates	ETR			MIRU profile*	Additional VNTR loci					
			A	B	C		0424	1955	1982	2163b	2401	3690
1	Household contact	2	–	–	–	225425173433	5	3	8	0	2	4
2	Community outbreak	6	–	–	–	224325153323	4	3	9	2	4	5
3	Work contacts	3	3	2	3	225313153323	1	2	5	4	4	6
4	Reactivation in a patient	2	4	2	5	223225153422	1	2	5	2	2	3
5	Patient/carer	2	2	2	4	224323163213	4	2	2	3	2	2
6	Community outbreak	2	2	2	5	223125153323	2	1	5	2	2	6
7	Community outbreak	2	4	2	4	223325173533	4	4	6	5	4	4
8	Family contacts	2	–	–	–	225323153223	2	2	6	3	3	4
9	Laboratory cross-contamination	2	3	2	2	224325153323	2	3	8	3	4	6
10	Laboratory cross-contamination	3	–	–	–	225125113322	1	2	6	0	4	4
11	Reactivation in a patient	2	3	2	1	223225143324	2	2	7	1	4	4
12	Family contacts	2	3	2	3	225313153323	2	1	6	6	4	4
13	Reactivation in a patient	2	4	1	5	223315143423	2	2	5	1	2	4
14	Social contacts	2	–	2	–	224323163213	4	2	2	3	2	2
15	Public house outbreak	8	–	–	–	223125153324	2	1	4	0	2	8
16	Laboratory cross-contamination	6	3	2	3	225323153323	2	2	6	5	3	4
17	Patient contacts	2	3	2	4	223125163324	2	1	5	3	2	6
18	Laboratory cross-contamination	2	2	2	4	224323163213	4	2	2	3	2	2
19	Community outbreak	3	2	2	4	224328153324	4	3	2	2	2	2
20	Reactivation in a patient	2	5	2	2	226325173543	4	3	8	0	2	2

*Order of MIRU loci: 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40.

at the locus and n is the number of isolates, and $n/(n–1)$ is a correction for bias in small samples.[19] The HGDI was calculated as described by Hunter and Gaston.[20]

Results

Selection of additional VNTR loci

Six loci were selected which had previously been demonstrated to have ADI values >0.6 according to Supply *et al*.[21] The loci were chosen with the expectation to display variability in unrelated isolates, stability in clonally related isolates (i.e., epidemiological linked) and potential for robust PCR amplification.[16] The 15-loci subset that Supply *et al*.[21] has proposed as the new standard for first-line routine epidemiological discrimination of *M. tuberculosis* isolates includes six of the 12 original VNTR loci, plus two ETRs and five of the six additional loci studied herein.

Comparison of gel electrophoresis and non-dHPLC for sizing PCR products generated from the VNTR loci

All 100 products analysed using agarose gel electrophoresis were scored with exactly the same number of VNTRs per isolate as interpreted by the WAVE non-dHPLC; hence, this detection method was demonstrated to have 100% reproducibility. The reproducibility and accuracy of sizing DNA fragments was also checked and standardised by analysing DNA fragments from *M. tuberculosis* H37Rv as a positive control. This consistently gave the expected amplicon size and VNTR copy number for each locus.

Application of the additional VNTR loci to epidemiologically linked isolates

Genotyping using 15 MIRU-VNTR loci had previously clustered 57 isolates into 20 groups (known as clusters 1–20, Table 2) with established epidemiological links. When the same 57 epidemiologically linked *M. tuberculosis* isolates were anonymised and then typed using the six additional VNTR loci, they remained grouped in the 20 clusters and hence demonstrated a level of stability suitable for accurate, epidemiologically relevant clustering.

Application of the additional VNTR loci to clusters of isolates with common MIRU-VNTR profiles

One hundred and fifty DNA samples extracted from *M. tuberculosis* isolates by the HPA Regional Reference Centre for Mycobacteriology (2002–2007) were included in this study. Previous MIRU-VNTR typing using 15 loci had grouped these strains into six clusters representing the six most common MIRU-VNTR profiles, with up to 30 isolates in each cluster. Clustered isolates shared the same MIRU-VNTR profile, yet had no proven epidemiological links. All six common profile clusters previously identified using the 15 loci technique were split into subclusters when tested with the six additional VNTR loci (Table 3). The largest cluster size was 14 (9.3%) when typed using the six

Table 3. Application of additional VNTR loci to clusters of isolates with common MIRU-VNTR profiles.

Common 15 loci cluster	ETR A	ETR B	ETR C	MIRU profile	No. isolates	No. additional loci subclusters
1	3	2	3	225313153323	30	14
2	4	2	2	225425173533	30	6
3	4	2	2	226425173423	30	5
4	4	2	4	223325173533	25	14
5	3	2	4	224325153323	20	10
6	3	2	3	225325153323	15	7

additional VNTR loci, compared to 30 (20%) when typed using the original 15 MIRU-VNTR loci.

Allelic diversity and discriminatory power of the additional VNTR loci

Table 4 shows the allelic diversity (*h*) of the six additional VNTR loci based on the typing of 206 *M. tuberculosis* isolates. All but one of the additional VNTR loci were highly discriminative ($h>0.6$), with locus 2401 being only moderately discriminative ($h=0.5$).

The six common MIRU-VNTR-defined clusters in the unlinked set were subdivided into 56 different profiles when isolates were genotyped using the additional VNTR loci (Table 5). The discriminatory power of the method was calculated using the Hunter-Gaston discriminatory index (HGDI) equation[20] and an HGDI value of 0.952 was obtained.

Discussion

Molecular genotyping is increasingly being used to track infectious diseases as they spread in human populations.[22] Unlike many other diseases affecting the developing world, TB can be treated and controlled[2] with the help of surveillance using molecular epidemiological approaches. *M. tuberculosis* genotyping can be used to monitor trends and outbreaks, to identify risk factors and gain better understanding of TB transmission, and for the evaluation and subsequent improvement of regional control programmes.[2]

The interpretation of all molecular epidemiological data is based on establishing whether a strain is identical to or different from other strains found within a study community.[13] Strains with shared genotypes are thought to represent ongoing transmission, while genotypically different strains are thought to be unrelated.[13,23,24] The information that genotyping provides can be very effective in evaluating and adapting the control measures for tuberculosis.[2,25]

The 15 loci technique previously used to genotype *M. tuberculosis* isolates at the HPA North East Regional Laboratory has demonstrated the existence of common profile clusters which may lead to the over-estimation of transmission rates. Using the six additional loci has demonstrated the existence of subclusters within the common profile clusters. Consequently, isolates previously thought to belong to the same cluster, and thus may represent ongoing transmission, are actually unrelated.

The MIRU-VNTR profiles of six sets of clustered isolates included in the unlinked set of this study were compared using the UK national database[26] of *M. tuberculosis* genotypes. All six of the common MIRU-VNTR profiles were found in the most common 15 national profiles, demonstrating that the study population is representative of the common *M. tuberculosis* population in the UK.

Supply *et al.*[21] defined the properties of ideal molecular markers to represent a compromise between i) sufficient variability to distinguish unrelated strains and ii) satisfactory clonal stability to identify isolates reliably from the same strains and trace transmission chains, as well as robustness in applicability to a wide range of strains. The *h* values found in this study correlate with those described by Supply *et al.*,[21] with no significant difference between these findings and those previously published.[27-29]

In the present study, the set of six additional MIRU-VNTR loci plus the current 15-loci method was found to provide the most accurate first-line molecular typing of *M. tuberculosis* when compared to use of the 15 loci alone. An HGDI value of 0.952 was demonstrated (Table 5) and, according to Hunter and Gaston,[20] an index of >0.90 is

Table 4. Allelic diversity of additional VNTR loci.

VNTR locus	Copy no. of repetitive unit(s)											Allelic diversity
	0	1	2	3	4	5	6	7	8	9	10	(*h*)
0424	0	23	63	8	82	30						0.71
1955	0	31	67	87	17	4						0.69
1982	0	0	21	2	10	28	34	10	74	16	11	0.80
2163b	20	72	27	18	28	34	7					0.80
2401	0	1	59	13	133							0.50
3690	0	0	13	7	110	46	22	0	8			0.65

Table 5. The discriminatory power of additional VNTR loci.

Total no. unrelated isolates	Total no. type patterns	No. unique types	No. clusters	No. clustered isolates (%)	Maximum no. isolates in a cluster	HGDI
149	56	36	20	113 (75.8%)	21	0.952

desirable if the typing results of a technique are to be interpreted with confidence.[20] Similar studies have shown varying HGDI results,[7,9–12,29] but in general a lower value for the previously used 15 MIRU-VNTR loci technique is seen (HGDI: 0.747),[30] with those incorporating the six additional VNTR loci showing higher values (HGDI: 0.995).[31]

Conversely, Kwara *et al.*[32] stated that genotypes based on just 12 MIRU loci are highly stable among epidemiologically linked isolates but sufficiently diverse to generate a resolution approaching that of IS6110. The high discriminatory power of this reported technique, together with its stability in epidemiologically linked isolates, reinforces the strength of the six additional MIRU-VNTR loci for increased resolution of *M. tuberculosis* genotyping in a routine setting.

This is the first time these six MIRU-VNTR loci have been applied to the fingerprinting of *M. tuberculosis* in the HPA North East Regional Laboratory. Although gel electrophoresis is a relatively simple and inexpensive technique used for amplicon size detection, it is labour-intensive, lacks accuracy, is subjective and is not suitable for large-scale analysis. This study has demonstrated that incorporating the WAVE as a high-throughput genotyping system provides an accurate method for analysing the additional six loci.

This study confirms that addition of loci 0424, 1982, 3690, 1955 and 2163b in the MIRU-VNTR genotyping of *M. tuberculosis* fulfils the criteria laid down by Supply *et al.*[21] and vastly increases the discriminatory capacity of this technique. However, locus 2401 only displays moderate discrimination and thus should not be included in future genotyping of isolates in this region. Among the different described sets of MIRU-VNTR loci, a system based on 15 loci is the most widely used in clinical mycobacteriology and for local outbreak investigation within the UK.

The ease of data exchange and comparison offered by the MIRU-VNTR genotyping method has made it the best alternative to IS6110 restriction fragment length polymorphism (RFLP) for typing large numbers of unknown *M. tuberculosis* strains, making it the new gold standard.[3,12,33] Nonetheless, this report demonstrates that even 15 MIRU-VNTR loci, when used alone, cause significant false clustering within common profile populations of *M. tuberculosis* and hence lack discriminatory power and adequate resolution. In order to overcome this, the discriminatory power of MIRU-VNTR genotyping must be increased. This study has validated a technique by which this can be achieved. □

The authors thank the Regional Reference Centre for Mycobacteriology within the Health Protection Agency (HPA) North East Regional Laboratory, Newcastle upon Tyne, UK, for supplying the TB isolate DNA. This work was funded by the Health Protection Agency.

References

1 Dye C, Williams BG. Eliminating human tuberculosis in the twenty-first century. *J R Soc Interface* 2008; **5**: 653–62.

2 Kanduma E, McHugh TD, Gillespie SH. Molecular methods for *Mycobacterium tuberculosis* strain typing: a users' guide. *J Appl Microbiol* 2003; **94**: 781–91.

3 Sola C, Filliol I, Legrand E *et al*. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infect Genet Evol* 2003; **3**: 125–33.

4 Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. *J Clin Microbiol* 2001; **39**: 3563–71.

5 National TB Controllers Association/CDC Advisory Group on Tuberculosis Genotyping. *Guide to the application of genotyping to tuberculosis prevention and control*. Handbook for TB Controllers, Epidemiologists, Laboratorians, and Other Program Staff (online). Atlanta, GA: US Department of Health and Human Services, CDC, June 2004 (www.cdc.gov/TB/genotyping/manual.htm).

6 Cowan LS, Diem L, Monson T. Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States. *J Clin Microbiol* 2005; **43**: 688–95.

7 Mazars E, Lesjean S, Banuls AL *et al.* High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proc Nat Acad Sci USA* 2001; **98**: 1901–6.

8 Savine E, Warren RM, van der Spuy GD *et al*. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2002; **40**: 4561–6.

9 Blackwood KS, Wolfe JN, Kabani AM. Application of mycobacterial interspersed repetitive unit typing to Manitoba tuberculosis cases: can restriction fragment length polymorphism be forgotten? *J Clin Microbiol* 2004; **42**: 5001–6.

10 Hawkey PM, Smith EG, Evans JT *et al.* Mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* compared to IS6110-based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. *J Clin Microbiol* 2003; **41**: 3514–20.

11 Gibson A, Brown T, Baker L, Drobniewski F. Can 15-locus mycobacterial interspersed repetitive unit-variable-number tandem repeat analysis provide insight into the evolution of *Mycobacterium tuberculosis*? *Appl Environ Microbiol* 2005; **71** (12): 8207–13.

12 Gopaul KK, Brown TJ, Gibson AL, Yates MD, Drobniewski FA. Progression toward an improved DNA amplification-based typing technique in the study of *Mycobacterium tuberculosis* epidemiology. *J Clin Microbiol* 2006; **44**: 2492–8.

13 van Embden JDA, Cave MD, Crawford JT *et al*. Strain

identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; **31**: 406–9.
14 www.ncbi.nlm.nih.gov/Genbank/index.html
15 Lasergene (Microsoft Windows version 7).
16 van Deutekom H, Supply P, de Haas PE *et al*. Molecular typing of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit-variable number tandem repeat analysis, a more accurate method for identifying epidemiological links between patients with tuberculosis. *J Clin Microbiol* 2005; **43**: 4473–9.
17 www.ncbi.nlm.nih.gov/blast/Blast.cgi.html
18 Evans JT, Hawkey PM, Smith EG, Boese KA, Warren RE, Hong G. Automated high-throughput mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* strains by a combination of PCR and nondenaturing high-performance liquid chromatography. *J Clin Microbiol* 2004; **42**: 4175–80.
19 Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* 1986; **51**: 873–4.
20 Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's Index of Diversity. *J Clin Microbiol* 1988; **26**: 2465–6.
21 Supply P, Allix C, Lesjean S *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; **44**: 4498–510.
22 Kato-Maeda M, Rhee JT, Gingeras TR *et al*. Comparing genomes within the species *Mycobacterium tuberculosis*. *Genome Res* 2001; **11**: 547–54.
23 Narayanan S. Molecular epidemiology of tuberculosis. *Indian J Med Res* 2004; **120**: 233–47.
24 Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN. Molecular epidemiology of tuberculosis: current insights. *Clin Microbiol Rev* 2006; **19**: 658–85.
25 Alland D, Kalkut GE, Moss AR *et al*. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. *N Engl J Med* 1994; **330**: 1710–6.
26 www.hpa-bionum.org.uk/TBtyping
27 Smittipat N, Billamas P, Palittapongarnpim M *et al*. Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. *J Clin Microbiol* 2005; **43**: 5034–43.
28 Surikova OV, Voitech DS, Kuzmicheva G *et al*. Efficient differentiation of *Mycobacterium tuberculosis* strains of the W-Beijing family from Russia using highly polymorphic VNTR loci. *Eur J Epidemiol* 2005; **20**: 963–74.
29 Yokoyama E, Kishida K, Uchimura M, Ichinohe S. Improved differentiation of *Mycobacterium tuberculosis* strains, including many Beijing genotype strains, using a new combination of variable number of tandem repeats loci. *Infect Genet Evol* 2007; **7**: 499–508.
30 Nikolayevskyy V, Gopaul K, Balabanova Y, Brown T, Fedorin I, Drobniewski F. Differentiation of tuberculosis strains in a population with mainly Beijing-family strains. *Emerg Infect Dis* 2006; **12**: 1406–13.
31 Alonso-Rodríguez N, Martínez-Lirola M, Herránz M *et al.* Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in *Mycobacterium tuberculosis* molecular epidemiology studies. *BMC Microbiol* 2008; **8**: 34–42.
32 Kwara A, Schiro R, Cowan LS *et al*. Evaluation of the epidemiologic utility of secondary typing methods for differentiation of *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2003; **41**: 2683–5.
33 Oelemann MC, Diel R, Vatin V *et al*. Assessment of an optimized mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. *J Clin Microbiol* 2007; **45**: 691–7.