Utility of mycobacterial interspersed repetitive unit typing for differentiating *Mycobacterium tuberculosis* isolates in Wuhan, China

Hui Han,^{1,5} Fang Wang,² Yong Xiao,³ Yi Ren,³ Yanjie Chao,⁴ Aizhen Guo⁴ and Lingxiang Ye¹

Correspondence

Lingxiang Ye

yelx2004@163.com

¹Department of Epidemiology, School of Public Health, Huazhong University of Science and Technology, Wuhan 430030, China

²Department of Social Medicine, School of Public Health, Huazhong University of Science and Technology, Wuhan 430030, China

³Wuhan Tuberculosis Prevention and Treatment Hospital, Wuhan 430030, China

⁴National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China

⁵National Center for Women and Children's Health, China CDC, Beijing 100013, China

Mycobacterial interspersed repetitive unit (MIRU) typing has been found to allow rapid, reliable, high-throughput genotyping of *Mycobacterium tuberculosis*, and may represent a feasible approach to study *M. tuberculosis* molecular epidemiology. To evaluate the use of MIRU typing in discriminating *M. tuberculosis* strains, isolates from 105 patients in Wuhan City, China, were genotyped by this method as compared to spoligotyping. MIRU typing identified 55 types that defined 21 clusters and 34 unique isolates. The discriminatory power was high [Hunter–Gaston discriminatory index (HGDI), 0.97]. Spoligotyping showed that 86 (81.9%) of 105 isolates belonged to the Beijing family genotype. For Beijing family and non-Beijing strains, the discriminatory power of MIRU was high (HGDI, 0.95 and 0.98, respectively). Among the alleles of the MIRU loci for the Beijing family, only locus 26 was highly discriminative, but for non-Beijing strains, loci 10, 16 and 26 were highly discriminative. MIRU typing is a simple and fast method which may be used for preliminary screening of *M. tuberculosis* isolates in China.

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INTRODUCTION

Despite global efforts to combat *Mycobacterium tuberculosis*, it remains a major public health problem worldwide, especially in developing countries such as China. Infection with *M. tuberculosis* is latent, therefore the methods of classical epidemiology cannot reveal disease prevalence. DNA fingerprinting of *M. tuberculosis* isolates is a powerful tool for studying the molecular epidemiology of tuberculosis (TB). RFLP analysis based on the variation in copy number of an *M. tuberculosis*-specific insertion sequence, IS6110, is a powerful tool for studying molecular epidemiology, and is considered a 'gold standard' for DNA fingerprinting of *M. tuberculosis* (van Embden *et al.*, 1993; Kremer *et al.*, 1999). The procedures involved, however, are time-consuming and technically demanding, and require large quantities of DNA. For low-copy-number

strains (fewer than five IS6110 copies), it is a less reliable indicator of clonality and therefore a poor predictor of epidemiological relationships (Kamerbeek *et al.*, 1997; Warren *et al.*, 1996). A PCR-based typing method using variable number tandem repeats (VNTRs), which are mycobacterial interspersed repetitive units (MIRUs) in 12 human minisatellite-like regions of the *M. tuberculosis* genome, has been developed and has produced some promising initial results (Supply *et al.*, 2000, 2001). Studies have shown that the discriminatory power of this method is comparable to that of IS6110 RFLP (Kremer *et al.*, 2005). Moreover, MIRU typing has also shown its usefulness in studying the population structure of *M. tuberculosis* (Sola *et al.*, 2003; Supply *et al.*, 2003). Due to its high resolution, simplicity, sensitivity, high reproducibility and easy inter-laboratory comparison (Kanduma *et al.*, 2003; Mazars *et al.*, 2001; Mokrousov *et al.*, 2004; Kam *et al.*, 2005), the 12-locus MIRU typing method has been found to be highly suitable for global epidemiological surveillance of TB. In some studies, MIRU typing has even been found to produce more distinct patterns than IS6110 RFLP (Barlow *et al.*, 2001; Cowan *et al.*, 2002; Sun *et al.*, 2004).

Abbreviations: HGDI, Hunter–Gaston discriminatory index; MIRU, mycobacterial interspersed repetitive unit; TB, tuberculosis; VNTR, variable number tandem repeat; WTPH, Wuhan Tuberculosis Prevention and Treatment Hospital.

The aim of this study was to investigate the differentiation ability of MIRU typing and spoligotyping. Spoligotyping is the 'gold standard' method for identifying Beijing strains (Bifani *et al.*, 2002; Glynn *et al.*, 2002; Mokrousov *et al.*, 2003), which bear the characteristic nine-spacer reaction on spacers 35–43, and show an absence of hybridization to spacers 1–34 (van Soolingen *et al.*, 1995; Kremer *et al.*, 2004). Therefore, spoligotyping was employed to identify the Beijing genotype before MIRU in this study. This will be helpful for assessing the feasibility of appropriate methods in TB epidemiological studies in China.

METHODS

Mycobacterial strains and genomic DNA. A total of 105 cases of TB constituted 20 % of the 525 patients during the study period. Samples were collected randomly from in-patients at Wuhan Tuberculosis Prevention and Treatment Hospital (WTPTH) from July to December 2005. Wuhan City is one of the largest in central China. WTPTH is a unique facility for the diagnosis and treatment of TB, and all the TB patients accepted treatment in this hospital. The genomic DNA of the studied strains was isolated according to the recommended method (van Embden *et al.*, 1993). *M. tuberculosis* H37Rv was used as a control. To test the reproducibility of MIRU typing, we blindly typed 20 isolates in two independent experiments.

Spoligotyping. Spoligotyping was performed with a commercial spoligotyping kit (Isogen Bioscience), according to the instructions supplied by the manufacturer. Briefly, template DNA was amplified with 20 pmol biotinylated DRa primer and DRb primer for 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 30 s. Subsequently, the PCR products were heat-denatured and applied to the blot using Miniblotter MN45 (Immuntics). Following the hybridization, the membrane was incubated with horseradish peroxidase–streptavidin conjugate (Zymed). Hybridization signals were detected by enhanced chemiluminescence (Pierce) with a luminocapture instrument (Kodak Image Station 4000MM). Spoligotyping detected the presence of 43 spacer sequences in the direct repeat region by reversed-line blot hybridization (Kamerbeek *et al.*, 1997). The Beijing family genotype was identified by its unique spoligotyping pattern.

MIRU. MIRU analysis was performed essentially as described by Kwara *et al.* (2003) with the 2 × Taq PCR MasterMix kit (TIANGEN) for the PCR. The amplification parameters consisted of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension at 72 °C for 7 min in an iCycler iQ Multicolor Real-Time PCR detection system (Bio-Rad). The PCR products were analysed on a 2 % agarose gel. The sizes of the amplicons were estimated by comparing with 50 and 100 bp DNA ladders (TIANGEN). The MIRU copy number per locus was calculated as described by Kwara *et al.* (2003).

Statistical analysis. The MIRU allelic diversity (h) at each of the 12 loci was calculated by the equation $h = 1 - \sum x_i^2 / [n(n-1)]$, where x_i is the frequency of the i th allele at the locus, and n is the number of isolates (Graur & Li, 2000). The Hunter–Gaston discriminatory index (HGDI) (Hunter & Gaston, 1988) was used as a numerical index for MIRU discriminatory power. HGDI was calculated by using the formula

$$\text{HGDI} = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j-1) \right]$$

where N is the total number of strains in the typing scheme, s is the total number of different MIRU–VNTR patterns, and n_j is the number of strains belonging to the j th pattern.

RESULTS AND DISCUSSION

Reproducibility of MIRU typing

In order to check the reproducibility of MIRU typing, we performed two independent blind typing tests with 20 isolates. The results were identical between the two tests, which indicates that MIRU typing had 100 % reproducibility under our conditions.

Genetic diversity of *M. tuberculosis* isolates in Wuhan

Using spoligotyping and MIRU typing, we analysed 105 *M. tuberculosis* isolates from 105 patients. The digital result of the Beijing family spoligotyping genotype is 000000000003771, as described by Dale *et al.* (2001). Spoligotyping identified 17 types that defined three clusters (91 isolates) and 14 unique isolates. The discriminatory power was 0.33 (HGDI, 0.33). The largest cluster was Beijing family isolates (86 isolates, 81.9 %), which was dominant in Wuhan City. The others were non-Beijing isolates (19 isolates), which defined 13 clusters (HGDI, 0.96).

MIRU typing identified 55 types that defined 21 clusters and 34 unique isolates (HGDI, 0.970; Table 1). Forty types of Beijing family isolates (86 isolates) were identified by MIRU; these defined 17 clusters and 23 unique isolates, with the HGDI being 0.95. The largest cluster was 2232-2517-3533, with 14 members, followed by 2233-2517-3533, 2223-2517-3533 and 2222-1417-3533, with 11, 4 and 4 members, respectively. The non-Beijing isolates (19 isolates) defined 15 types, 4 clusters and 11 unique isolates (HGDI, 0.98). Members of the Beijing family constitute a homogeneous group of *M. tuberculosis*, as demonstrated by their highly similar RFLP patterns and conserved family-specific spoligotyping pattern. This homogeneity was also observed in MIRU typing. For non-Beijing isolates, the HGDI for MIRU and spoligotyping was 0.98 and 0.96, respectively, and the difference between the values was small. However, for Beijing family isolates, the HGDI was 0.95 and 0, respectively, and the difference was large.

Allelic diversity of the MIRU loci

Table 2 shows the allelic diversity of the 12 MIRU loci, based on this collection of samples. Loci 10, 16 and 26 were highly discriminative (≥ 0.6), loci 23, 31 and 40 were moderately discriminative (≥ 0.3), and loci 2, 4, 20, 24, 27 and 39 were poorly discriminative (< 0.3) when ranked according to Sola *et al.* (2003). There were some differences between Beijing family and non-Beijing isolates. For Beijing family isolates, only locus 26 was highly discriminative, which supports previous findings (Mokrousov *et al.*, 2004;

Table 1. MIRU and spoligotyping cluster for 105 *M. tuberculosis* isolates

MIRU pattern*	No. of isolates	Spoligotyping pattern	MIRU pattern*	No. of isolates	Spoligotyping pattern
2232-2517-3533	14	000000000003771	2212-2517-3533	1	000000000003771
2233-2517-3533	11	000000000003771	2221-2511-3522	1	767777777760731
2223-2517-3533	4	000000000003771	2222-1417-3632	1	000000000003771
2222-1417-3533	4	000000000003771	2222-2515-3533	1	000000000003771
2222-2518-3533	3	000000000003771	2222-2516-3324	1	757777777760771
2232-2516-3533	3	000000000003771	2222-2516-3533	1	000000000003771
2233-2515-3533	3	000000000003771	2222-2517-3533	1	000000000003771
2233-2518-3533	3	000000000003771	2223-2516-3222	1	577000777760771
2133-2518-3533	2	000000000003771	2223-2517-3424	1	000000000003771
2212-2417-3533	2	000000000003771	2223-2517-3433	1	000000000003771
2222-1417-2533	2	000000000003771	2223-2616-3323	1	577777777760731
2222-1418-3533	2	000000000003771	2224-1417-3323	1	577777777760771
2222-2513-3533	2	000000000003771	2232-1417-3533	1	000000000003771
2223-2516-3532	2	000000000003771	2222-2417-3533	1	777777777760700
2232-2518-3533	2	000000000003771	2522-2511-3325	1	577777777760731
2234-2518-3533	2	000000000003771	2232-2515-3533	1	000000000003771
2241-2411-3222	2	777777777760771	2232-2517-3733	1	000000000003771
		777737777760771			
2241-2511-3322	2	777777777760700	2232-2518-3433	1	000000000003771
2412-2514-2322	2	777757777760771	2233-2516-2534	1	000000000003771
		767777777760731			
2422-2515-2322	2	777757777760731	2233-2517-3433	1	000000000003771
		767757777760771			
2233-25110-3533	2	000000000003771	2233-2518-2531	1	000000000003771
2121-1517-3533	1	000000000003771	2233-2616-3533	1	000000000003771
2141-1411-3222	1	777777777760771	2234-2517-3333	1	000000000003771
2212-1417-3333	1	000000000003771	2234-2518-3233	1	000000000003771
2212-2315-3322	1	676763777776000	2243-2516-3533	1	000000000003771
2212-2414-2222	1	757737777760771	2433-2517-3533	1	000000000003771
2212-2515-3222	1	777737777760771	2243-15110-3433	1	000000000003771
2213-2517-3325	1	000000000003771			

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

Nikolayevskyy *et al.*, 2006), loci 10 and 16 were moderately discriminative, and the others were poorly discriminative. Only 25 % of 12 loci were more than moderately discriminative. However, for non-Beijing isolates, loci 10, 16 and 26 were highly discriminative, loci 4, 23, 27, 31 and 40 were moderately discriminative, and the others were poorly discriminative. About 66.7 % of 12 loci had an allelic diversity greater than 0.3. The difference was significant ($P=0.041$). This observation implied a relatively greater diversity of genetic makeup of the non-Beijing strains.

In this study, we described the use of MIRU typing as compared with spoligotyping of *M. tuberculosis* isolates. The discriminatory power (HGDI) for MIRU typing and spoligotyping was 0.97 and 0.33, respectively. It was found that the discriminatory power of the two methods for 19 non-Beijing isolates was almost equal (HGDI, 0.98 and 0.96). This finding was different from those of other studies (Sola *et al.*, 2003). The small number of non-Beijing isolates might be the reason for this difference. In our study, there was 100 % reproducibility in MIRU typing.

Table 2. Allelic diversity of each MIRU locus

Alleles*	No. of isolates with MIRU locus											
	2	4	10	16	20	23	24	26	27	31	39	40
H_T	0	0.15	0.61	0.60	0.24	0.34	0	0.70	0.15	0.43	0.29	0.41
H_B	0	0.08	0.52	0.55	0.25	0.25	0	0.60	0.08	0.23	0.03	0.23
H_{N-B}	0	0.38	0.70	0.69	0.14	0.59	0	0.76	0.35	0.59	0.14	0.39

* H_T , allelic diversity of 105 *M. tuberculosis* isolates; H_B , allelic diversity of 86 Beijing family isolates; H_{N-B} , allelic diversity of 19 non-Beijing strains.

According to the reports by Kremer *et al.* (1999) and Supply *et al.* (2000), MIRU typing was also found to be 100% reproducible, sensitive and specific for *M. tuberculosis* isolates, a performance that has not been achieved by any other typing method tested under the same conditions.

Most Beijing strains are prevalent in China, where the TB disease burden is high and resources are limited, therefore only an affordable method should be used for typing. This means that efforts must be increased to improve the methodology and cut down costs. MIRU typing based on a capillary electrophoresis system offers convenience, automation, a short turnaround time, high throughput and perfect reproducibility, and may be the method of choice for *M. tuberculosis* strain typing in this country.

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