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Genetic Diversity and Drug Resistance of 133 *Mycobacterium tuberculosis* Isolates from Jiangxi Province, China

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Abstract

The genotypes of *Mycobacterium tuberculosis* (*M. tuberculosis*) have been found to differ in their resistance to different drugs. Although there is a high incidence of tuberculosis (TB) in Jiangxi, knowledge of the genotypes of *M. tuberculosis* in this province is limited in recent years. In this study, we investigated the relationship between genetic diversity and drug resistance in *M. tuberculosis* isolates collected from Jiangxi during January to October, 2014. A total of 133 *M. tuberculosis* isolates collected from the Jiangxi Chest Hospital were genotyped using both spacer oligonucleotide typing (spoligotyping) and 24-locus mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR). The resistance of these isolates to four first-line, and four second-line, anti-TB drugs, namely, isoniazid, rifampicin, ethambutol, streptomycin, capreomycin, amikacin, levofloxacin and protionamide, was then tested. The results indicate that the Beijing family was the most prevalent genotype (75.94%), followed by the T1 family (13.53%), the MANU2 family (1.50%), the T2 family (0.75%) and the Beijing-like genotype (0.75%). We also found nine new genotypes that did not match those in the Spoldb4.0 database. The 24-locus MIRU-VNTR method had a low clustering rate (10.53%) and a high HGDI (0.9723), and proved a high resolution method for genotyping *M. tuberculosis* isolates. More than half of the *M. tuberculosis* isolates collected in the present study were resistant to anti-TB drugs (56.39%, 75/133), and the majority of these were resistant to more than one drug (81.33%, 61/75). The Beijing family is the most prevalent TB strain in Jiangxi province, China. More than half the *M. tuberculosis* isolates collected were resistant to anti-TB drugs, and the majority was resistant to more than one drug. There was, however, no relationship between genetic diversity and drug resistance. Moreover, our results suggest that treatment history can lead to the development of drug resistance ($P < 0.05$), which supports the more moderate use of medication in the treatment of TB.

Keywords: *Mycobacterium tuberculosis*; Genotyping; Spoligotyping; MIRU-VNTR; Drug susceptibility

Abbreviations: *M. Tuberculosis*: *Mycobacterium Tuberculosis*; TB: Tuberculosis; MIRU-VNTR: Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats; RFLP: Restriction Fragment Length Polymorphism; MDR: Multidrug-Resistant; XDR: Extensively Drug-Resistant; DST: Drug Susceptibility Testing; INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol; SM: Streptomycin; CAP: Capreomycin; AMK: Amikacin; LVFX: Levofloxacin; TH1321: Protionamide; HGDI: Hunter-Gaston Discriminatory Index. R: Resistance; S: Sensitive

Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*) [1-3]. TB's human mortality rate is second only to that of HIV/AIDS; of the estimated 9.6 million people who developed TB in 2014, 1.5 million died from the disease. 58% of the 9.6 million TB cases recorded in 2014 occurred in South-East Asia and the Western Pacific, and India and China accounted for 23% and 10% of these cases, respectively [4]. In China, the incidence and prevalence of TB differs from province to province. The increase in TB strains that are resistant to both isoniazid and rifampicin (MDR), and those resistant to isoniazid and rifampicin, plus fluoroquinolone and a second-line injectable agent (XDR), inhibits the treatment of the disease [5-7]. An estimated 3.3% of new and 20% of previously treated, TB cases in 2014 involves a kind of MDR-TB. On average, an estimated 9.7% of patients with MDR-TB have XDR-TB, which makes treatment more challenging, especially in developing countries. Over half of all MDR-TB cases occur in just three countries; India, China and the Russian Federation [4,8,9].

Molecular typing of *M. tuberculosis* strains has proven to be a valuable tool for tracking the transmission chain and detecting suspected outbreaks [10,11]. In the past decade, DNA fingerprinting based on restriction fragment length polymorphism (RFLP) of IS*6110* insertion sequences has been the gold standard for typing *M. tuberculosis* strains. Unfortunately, this method is labor intensive, time-consuming, and requires large quantities of DNA. Moreover, it can fail to discriminate between isolates with low IS*6110* copy numbers [11-14]. A number of PCR-based methods have been developed to solve these problems, such as spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR). Combining these two methods can achieve a level of discrimination comparable to that obtained by RFLP [15-17].

Jiangxi province is located in southeastern China on the middle and lower reaches of the Yangtze River. It has a total area of 16.69 square kilometers and had a population of 44.57 million in 2010. According to the National Fourth Tuberculosis Epidemiology Investigation, the infectious TB prevalence is 2036/million in Jiangxi compared with the national average of 1580/million. Yuan et al. [18] have conducted a study on patients with MDR and XDR TB at the Jiangxi Chest Hospital from July 2010 to June 2011 [19]. The present research attempts to launch

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a systematic study of the prevalence of the disease by identifying the predominant TB genotypes in Jiangxi Chest Hospital during January to October 2014, and assessing and comparing the drug resistance of these genotypes.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. All patients in the study signed informed consent. Ethics respected throughout the study period.

Mycobacterial isolates

A total of 154 clinical *M. tuberculosis* samples were collected from 154 pulmonary TB patients at the Jiangxi Chest Hospital between January to October, 2014. All patients from which samples were collected were both sputum microscopy positive and culture positive. Among them, there were only 133 samples were genotyping successfully which were collected 3 were from Fuzhou, 7 were from Ganzhou, 5 from Ji'an, 3 from Jingdezhen, 5 from Jiujiang, 57 from Nanchang, 21 from Shangrao, 3 from Xinyu, 8 from Yichun, 4 from Yingtan, and 17 did not register an address.

Genomic DNA extraction

Genomic DNA was extracted by boiling a cultured cell suspension scraped from Lowenstein-Jensen slants into 200 ml distilled water at 85°C for 30 min. The supernatant containing the DNA was then collected by centrifugation at 12000 rpm for 10 min. The centrifuged supernatant was transferred to a clean tube and stored at -20°C for future use [20,21].

Locus name	Alias(es)	PCR primer pairs(5'-3')	Repeat unit length (bp)	Predicted length in H37Rv	Copy number in H37Rv
H37Rv-2165	ETR-A	F: ATTTCGATCGGGATGTTGAT R: TCGGTCCCATCACCTTCTTA	75	397	3
H37Rv-2461	ETR-B	F: GCGAACACCAGGACAGCATCATG R: GGCATGCCGGTGATCGAGTGG	57	292	3
H37Rv-0577	ETR-C	F: GACTTCAATGCGTTGTTGGA R: GTCTTGACCTCCACGAGTGC	58	346	4
H37Rv-0580	ETR-D MIRU 4	F: GCGCGAGAGCCCGAACTGC R: GCGCAGCAGAA ACGTCAGC	77	330	3
H37Rv-3192	ETR-E MIRU 31	F: ACTGATTGGCTTCATACGGCTTTA R: GTGCCGACGTGGTCTTGAT	53	651	3
H37Rv-0154	MIRU 02	F: TGGACTTGCAGCAATGGACCAACT R: TACTCGGACGCCGGCTCAAAAT	53	508	2
H37Rv-0960	MIRU 10	F: GTTCTTGACCAACTGAGTCGTCC R: GCCACCTTGGTGATCAGCTACCT	53	643	3
H37Rv-1644	MIRU 16	F: TCGGTGATCGGGTCCAGTCCAAGTA R: CCCGTCGTGCAGCCCTGGTAC	53	671	2
H37Rv-2059	MIRU 20	F: TCGGAGAGATGCCCTTCGAGTTAG R: GGAGACCGCGACCAGGTACTTGTA	77	591	2
H37Rv-2531	MIRU 23	F: CAGCGAAACGAACTGTGCTATCAC R: CGTGTCCGAGCAGAAAAGGGTAT	53	873	6
H37Rv-2687	MIRU 24	F: CGACCAAGATGTGCAGGAATACAT R: GGGCGAGTTGAGCTCACAGAA	54	447	1
H37Rv-2996	MIRU 26	F: CCCGCCTTCGAAACGTCGCT R: TGGACATAGGCGACCAGGCGAATA	51	613	3
H37Rv-3007	MIRU 27 QUB 5	F: TCGAAAGCCTCTGCGTGCCAGTAA R: GCGATGTGAGCGTGCCACTCAA	53	657	3
H37Rv-4348	MIRU 39	F: CGCATCGACAAACTGGAGCCAAAC R: CGGAAACGTCTACGCCCCACACAT	53	646	2
H37Rv-0802	MIRU 40	F: GGGTTGCTGGATGACAACGTGT R: GGGTGATCTCGGCGAAATCAGATA	54	407	1
H37Rv-0424	Mtub04	F: CTTGGCCGGCATCAAGCGCATTATT R: GGCAGCAGAGCCCGGGATTCTTC	51	639	2
H37Rv-1955	Mtub 21	F: AGATCCCAGTTGTCGTCGTC R: CAACATCGCCTGGTTCTGTA	57	206	2
H37Rv-2347	Mtub 29	F: GCCAGCCGCCGTGCATAAACCT R: AGCCACCCGGTGTGCCTTGTATGAC	57	563	4
H37Rv-2401	Mtub 30	F: AGTCACCTTTCCTACCACTCGTAAC R: ATTAGTAGGGCACTAGCACCTCAAG	58	319	2
H37Rv-3171	Mtub 34	F: GGTGCGCACCTGCTCCAGATAA R: GGCTCTCATTGCTGGAGGGTTGTAC	54	488	3
H37Rv-3690	Mtub 39	F: A ATCACGGTAACTTGGGTTGTTT R: GATGCATGTTCGACCCGTAG	58	515	6
H37Rv-2163b	QUB 11b	F: CGTAAGGGGGATGCGGGAAATAGG R: CGAAGTGAATGGTGGCAT	69	412	5
H37Rv-4052	QUB 26	F: AACGCTCAGCTGTCGGAT R: CGGCCGTGCCGGCCAGGTCCTTCCCGAT	111	718	5
H37Rv-4156	QUB 4156	F: TGACCACGGATTGCTCTAGT R: GCCGGCGTCCATGTT	59	681	2

Table 1: Primer sequences of the 24-locus set used to identify *Mycobacterium tuberculosis* samples collected from TB patients.

Drug susceptibility testing (DST)

The Lowenstein-Jensen medium was impregnated with four first-line, and four second-line, anti-TB drugs; 1 μg/ml isoniazid (INH), 50 μg/ml rifampicin (RIF), 5 μg/ml ethambutol (EMB), 10 μg/ml streptomycin (SM), 10 μg/ml capreomycin (CAP), 10 μg/ml amikacin (AMK), 1μg/ml levofloxacin (LVFX) and 10 μg/ml protionamide (TH1321). If the area covered by a TB strain grown on a particular drug-impregnated medium was > 1% that of the same strain grown on a drug-free control culture, the strain was considered resistant to that particular drug. Conversely, if the area covered by a TB strain grown on a particular drug-impregnated medium was < 1% that of the control, the strain was considered sensitive to the drug [22]. DST was performed at the Jiangxi Chest Hospital.

Spoligotyping

Spoligotyping of samples was performed as described by Kamerbeek et al. [23]. The PCR-amplified biotin-labeled DR locus was hybridized against an array of 43 different immobilized DR spacers in a Miniblotter apparatus. The resulting hybridization signals were revealed by chemiluminescence and visualized as a profile of discrete dots. The profiles in binary format were entered into Excel spread-sheets and compared with those in the Spoldb4.0 database [24].

24-locus MIRU-VNTR PCR

The 24-locus PCR primers used in this study were described by Supply et al. and Li et al. (Table 1) [9,14,25]. DNA from *M. tuberculosis* H37Rv was used as a positive control. Each MIRU-VNTR locus was amplified individually in a 20 μl reaction volume, which was comprised of 10ul DNA polymerase enzyme mix (ComWin Biotech), 6 μl distilled water, 1ul former primer, 1 μl reverse primer, and 2 μl DNA template. The amplification cycle was 94°C 5 min followed by 35 cycles of 94°C 30s, 62°C 30 s, and 72°C 45 s, with a final 72°C 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel using a 100 bp DNA ladder marker (Table 1).

Data analysis

Genotyping patterns, including spoligotyping and MIRU-VNTR, were analyzed at http://www.miru-vntrplus.org [26]. The Hunter-Gaston discriminatory index (HGDI), which is used to evaluate the discriminatory power of the MIRU-VNTR method, was calculated at http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl, and using the following formula. N is the total number of strains. S is the total number of different strain types. nj is the number of strains belonging to the jth type. Associations among multiple categorical variables were evaluated with a two-tailed Chi-square test, P-values > 0.05 were considered statistically significant. All statistical analyses were conducted in SPSS 13.0 (SPSS Inc., USA).

$$HGDI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1} nj(jn-1) \right]$$

Results

Population description

The 133 patients from which TB samples were collected included 106 men, 26 women, and 1 of unknown gender. Five patients were < 20 years old, 30 were between 20 and 40, 55 between 40 and 60, 36 between 60 and 80, 6 were > 80, and 1 patient was of unknown age. Sixty-six patients were new cases, fifty had been previously treated, and the clinical history of 17 was unknown.

Results of spoligotyping and 24-locus MIRU-VNTR

123 of the 133 TB samples collected matched 5 identified genotypes

Binary[a]	Family[b]	SIT[c]	No[d]	Prevalence (%)
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing	1	99	74.43
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	Beijing	190	1	0.752
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■□□□■■■■■	Beijing	1162	1	0.752
□□■■■■■■■	Beijing-like	269	1	0.752
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	53	5	3.759
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□	T1	522	4	3.008
■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	334	2	1.504
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■□□□□■■■■■■■	T1	516	1	0.752
■■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	131	1	0.752
■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	498	1	0.752
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■	T1	888	1	0.752
■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	154	1	0.752
■■■■■■■■■■■■■■■□■■■■■□□■■■■■■■■■■□□□□■■■■■■■	T1	1053	1	0.752
■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	1105	1	0.752
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	52	1	0.752
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	MANU2	54	2	1.504
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■	New		1	0.752
■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	New		1	0.752
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□■□	New		1	0.752
■■■■■■□□■■■□■□■■□■■■□■■■■■■■■■■■□□■■■■■■■■■	New		1	0.752
■■■■■■□□■■■□■□■■■■■■□■■■■■■■■■■■□□■■■■■■■■■	New		1	0.752
■■■■■■□□■■■□■□■■□■■■□■■■■■■□■■■■■■□□■■■■■■■■■	New		1	0.752
■■■■■■□□■■■□■□□■□■■■□■■□□■■■■■■■■□□■■■■■■■■■	New		2	1.50
■□■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□■■■	New		1	0.752
■■■■■□□□■■■■■■■■■■■□□□□□□□□□■■■■□□□□■■□□■■■	New		1	0.752

[a] □, absence of spacer; ■, presence of spacer.
[b] Family matched from SpolDB4.0 database.
[c] SIT number from SpolDB4.0 database.
[d] Number of samples with a common SIT.

Table 2: Results of Spoligotyping 133 *Mycobacterium tuberculosis* samples collected from TB patients.

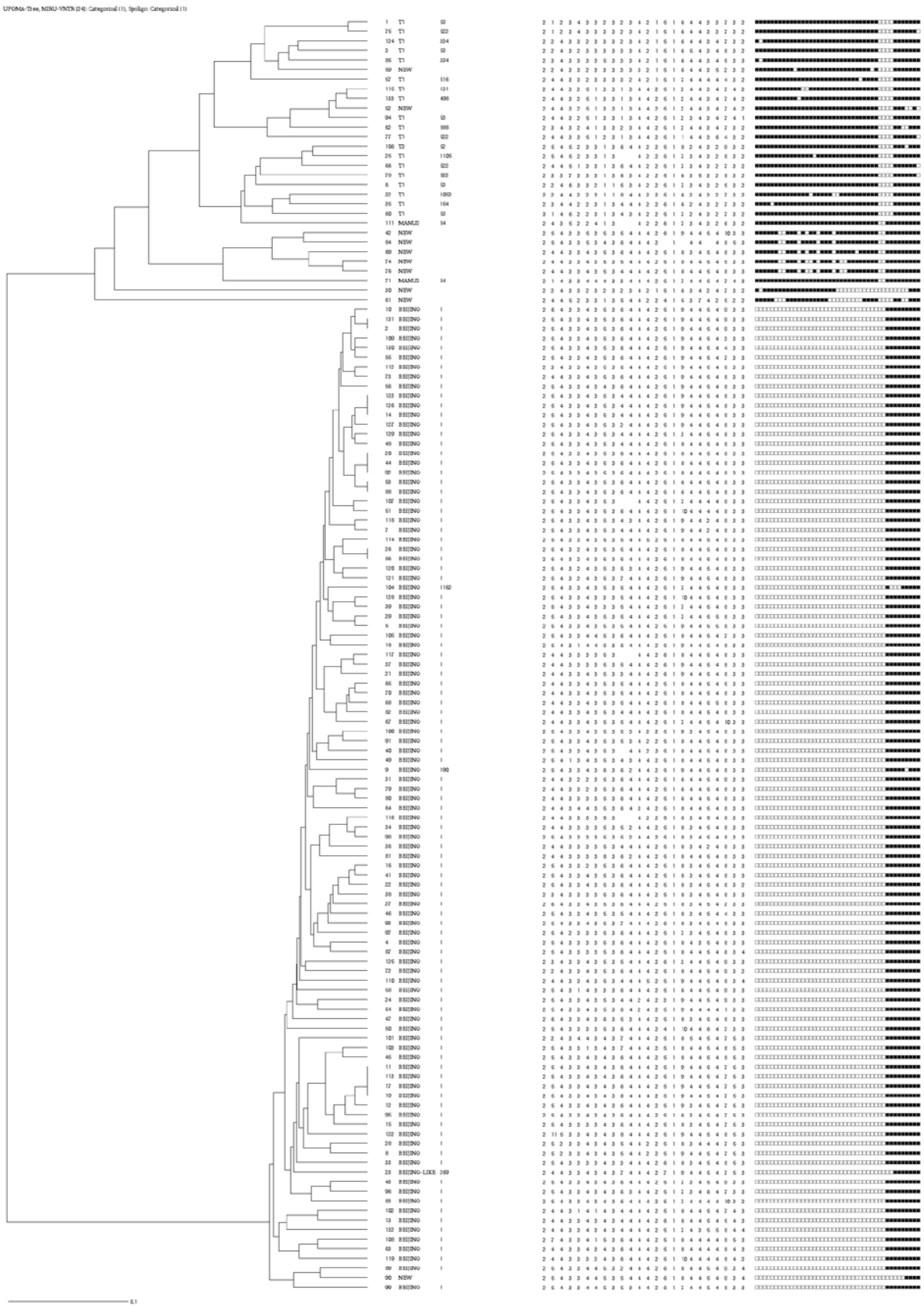

Figure 1: UPGMA-tree of the genotypes of 133 TB samples collected from TB patients based on spoligotyping and 24-locus MIRU-VNTR methods. From left to right; strain number, spoligotype family and SIT number based SpolDS4, MURU-VNTR pattern, and spoligotype pattern

in the SpolDB4.0 database; the remaining 10 could be classified into 9 unmatched, genotypes (Figure 1). The majority of the 123 matched samples clustered within the typical Beijing genotype (101 strains), followed by the T1 family (18 strains), MANU2 family (2 strains), T2 family (1 strain), and the Beijing-like genotype (1 strain) (Table 2). And the difference analyzed between Beijing and non- Beijing family were analyzed (Table 3). The Beijing and non-Beijing genotypes differed significantly in age composition (P = 0.043), but not in other demographic characteristics, including sex (P > 0.05), treatment history (P > 0.05) and smoking history (P > 0.05). All strains were genotyped using 24-locus, 15-locus and 12-locus MIRU-VNTR at http://www.miru-vntrplus.org, and 123 different VNTR genotypes of all strains were detected by the 24-locus method. 117 types were represented by single isolates and 16 genotypes had 2 to 4 strains. The clustering

Factors	Number (%)	Beijing family N (%)	non-Beijing family N (%)	χ^2	P
Sex					
Men	106 (80.30)	78 (59.09)	28 (21.21)		
Women	26 (19.70)	22 (16.67)	4 (3.03)	1.383	0.240
Age					
≤ 20	6 (4.55)	5 (3.79)	1 (0.76)		
20-40	31 (23.48)	29 (21.97)	2 (1.51)		
40-60	57 (43.18)	42 (31.82)	15 (11.36)		
60-80	33 (25.00)	20 (15.15)	13 (9.85)		
> 80	5 (3.79)	4 (3.03)	1 (0.76)	9.838	**0.043**
Treatment history					
No	66 (56.90)	49 (42.24)	17 (14.66)		
Yes	50 (43.10)	39 (33.62)	11 (9.48)	0.219	0.640
Smoking history					
No	57 (52.29)	43 (39.45)	14 (12.84)		
Yes	52 (47.71)	40 (36.70)	12 (11.01)	0.033	0.856

Table 3: Demographic features of patients infected with Beijing and non-Beijing family TB genotypes. P-values in bold type are statistically significant.

Locus	24	15	12	
MIRU2	X		X	
Mtub04	X	X		
ETR-C	X	X		
ETR-D	X	X	X	
MIRU40	X	X	X	
MIRU10	X	X	X	
MIRU16	X	X	X	
Mtub21	X	X		
MIRU20	X		X	
QUB11b	X	X		
ETR-A	X	X		
Mtub29	X			
Mtub30	X	X		
ETR-B	X			
MIRU23	X		X	
MIRU24	X		X	
MIRU26	X	X	X	
MIRU27	X		X	
Mtub34	X			
ETR-E	X	X	X	
Mtub39	X	X		
QUB26	X	X		
QUB4156	X	X		
MIRU39	X		X	
CR[a] of all strains	0.1053	0.1429	0.4211	
CR of Beijing family	0.0990	0.1386	0.4653	**P=0.001**
HGDI[b] of all strains	0.9723	0.9230	0.8692	
HGDI of Beijing family	0.9681	0.9147	0.8505	**P=0.017**

[a]CR: Clustering Rate
[b]HGDI: Hunter-Gaston discriminatory index

Table 4: Composition of different MIRU-VNTR loci sets and clustering rates of TB genotypes identified in samples collected from TB patients.

rates and HGDI of different loci varied significantly (Table 4) ($P < 0.05$), indicating that the discriminatory power of different MIRU-VNTR sets were significantly different. We found that the 24-locus MIRU-VNTR genotyping had the highest discriminatory power and therefore compared the results of this to those of previous studies (Table 5). MIRU-VNTR loci were further classified into high (> 0.6), moderate (0.3 to 0.6), or poor (< 0.3) discriminatory power based on their HGDI scores [26]. Only five loci (QUB11b, MIRU26, QUB26, Mtub04, Mtub21) had high discriminatory power, eight (MIRU10, ETR-E, MIRU27, ETR-A, MIRU39, QUB4156c, MIRU40, Mtub30) had moderate discriminatory power, and the last eleven (Mtub39, MIRU23, MIRU16, ETR-D, ETR-B, ETR-C, Mtub34, Mtub29, MIRU20, MIRU2, MIRU24) had poor discriminatory power. For the Beijing family, only two loci (QUB11b, MIRU26) had high discriminatory power (Tables 2, 3 and 4).

Drug susceptibility

A total of 133 TB samples were tested for drug resistance. Of these, 56.39% (75/133) were resistant to at least one drug and 43.61% (58/133) were sensitive to all four first-line anti-TB drugs. The proportion of drug resistance and the difference between Beijing and non-Beijing family strains in drug resistance are shown in Table 6. The highest resistance ratio was 48.65% (36/74) to CAP, and the lowest was 2.273% (3/132) to EMB. There were 7 MDR and 44 XDR strains among the 75 drug resistant strains. Moreover, another ten strains were AMDR, which is defined as resistant to two or more anti-TB drugs other than those involved in MDR or XDR resistance. Differences between Beijing and non-Beijing genotypes in drug resistance were not statistically significant ($P > 0.05$). The relationships between demographic variables and drug resistance are shown in Table 7. There was a significant association between treatment history and resistance to INH, RIF and LVFX ($P < 0.05$). Furthermore, treatment history also influenced the development of multiple drug resistance. Interestingly, there was also an association between smoking and resistance to INH and SM ($P < 0.05$). Hence, we also calculated the odds ratio of them to evaluate the risk of those factors. For treatment history, OR was 15.955 (95% confidence interval was 6.384 to 39.873) for INH, 14.250 (95% confidence interval was 5.786 to 35.097) for RIF and 6.667 (95% confidence interval was 1.366 to 32.536) for LVFX. For smoking, the OR was 0.385 (95% confidence interval was 0.177 to 0.837) for INH and 0.377 (95% confidence interval was 0.153 to 0.929) for SM.

Discussion

This is a systematic study of the molecular epidemiology of *M. tuberculosis* in Jiangxi Chest Hospital in recent years. Our results show that the Beijing family is the most predominant TB genotype which comprising 75.94% of all isolates. This is consistent with Yuan's study (78.9%) and results from other Chinese provinces where the Beijing genotype generally comprises between 66.67% and 91.17% of isolates. Indeed, with the exception of Guangdong where it comprises just 25.67% of isolates (a result that may be compromised by small sample size), the Beijing family comprises between 48.00% and 91.17% of TB isolates in all Chinese provinces for which the relevant data are available [19,20,39-41]. This is consistent with the results of another study found that the

VNTR Locus	All strains		Beijing family strains		Jiangxi Previous	Tibet	Gansu	Hong Kong	Hei-longji-ang	Shang hai	Bei jing	Jiang su	Inner Mongolia	Si chuan	Chong qing	Tian jin	Hubei Wuhan
	No. of alleles	HGDI	No. of alleles	HGDI	N = 123	N = 522	N = 409	N = 243	N = 179	N = 65	N = 211	N = 209	N = 318	N = 190	N = 198	N = 93	N = 86
QUB11b	9	0.764	8	**0.689**	**0.750**	**0.694**			**0.704**	**0.655**	**0.646**	**0.629**	**0.702**	**0.783**	**0.729**	**0.675**	
MIRU26	10	0.763	6	**0.692**	**0.830**	0.429	0.581	0.303	0.596	**0.612**	0.373	0.560	0.437	**0.727**	0.565	0.434	**0.600**
QUB26	10	0.645	9	0.542	**0.750**	0.525			**0.607**	0.595	0.570	**0.613**	0.572	**0.759**	**0.654**	0.504	
Mtub04	8	0.622	7	0.497	**0.610**	0.224			0.391	0.297	0.265	0.426	0.319	**0.722**	0.452		
Mtub21	7	0.606	5	0.443	**0.820**	0.491	**0.653**		0.396	0.523	0.393	0.535	0.574	**0.706**	**0.633**	0.397	
MIRU10	5	0.518	4	0.334	0.490	0.025	0.230	0.282	0.154	0.195	0.210	0.262	0.244	0.589	0.325	0.150	0.520
ETR-E	6	0.461	4	0.236	**0.760**	**0.617**	0.409	0.156	0.395	0.246	0.229	**0.668**	0.238	0.586	0.527	0.306	0.230
MIRU27	4	0.419	3	0.348		0.058	0.080	0.175		0.031	0.056	0.084	0.025	0.599	0.133	0.108	0.080
ETR-A	4	0.414	4	0.269	0.400	0.090	0.263		0.238	0.031	0.224	0.201	0.312	0.262	0.208	0.219	
MIRU39	4	0.387	3	0.150		0.147	0.090	0.356	0.290	0.286	0.127	0.178	0.132	**0.734**	0.254	0.109	0.030
QUB4156c	4	0.357	3	0.336	0.550	0.519			0.182	0.492	0.297	0.203	0.331	**0.605**	0.326		
MIRU40	4	0.335	4	0.186	0.430	0.221	0.276	0.409	0.292	0.147	0.171	0.276	0.221	0.558	0.299	0.210	0.230
Mtub30	3	0.322	3	0.150	0.490	0.033	0.125		0.133	0.091	0.065	0.196	0.149	0.140	0.166		
Mtub39	5	0.247	2	0.077	0.340	0.166	0.199		0.174	0.061	0.204	0.213	0.188	**0.626**	0.257		
MIRU23	6	0.244	4	0.200		0.033	0.057	0.110		0.061	0.179	0.250	0.037	0.176	0.096	0.130	0.250
MIRU16	4	0.234	4	0.133	0.480	0.158	0.398	0.080	0.200	0.242	0.265	0.262	0.126	**0.664**	0.204	0.088	0.560
ETR-D	7	0.172	3	0.039	**0.750**	0.066	0.090	0.072	0.212	0.061	0.152	0.536	0.086	0.185	0.144	0.066	0.080
ETR-B	3	0.128	2	0.020		0.029	0.034			0.000	0.038	0.056	0.037	0.031	0.144	0.022	
ETR-C	4	0.102	3	0.059	0.020	0.054	0.039				0.028	0.066	0.136	0.111	0.108	0.067	
Mtub34	4	0.059	2	0.039		0.029				0.089				0.120	0.050		
Mtub29	3	0.059	3	0.059		0.013			0.123	0.061				0.052	0.030		
MIRU20	2	0.030	1	0.000		0.440		0.008		0.061		0.010		0.031	0.087	0.000	0.250
MIRU2	2	0.015	1	0.000		0.000		0.000		0.000		0.008		0.000	0.010	0.000	0.000
MIRU24	1	0.000	1	0.000		0.000		0.000		0.000		0.000		0.000	0.000	0.000	0.000
Prevalence (%)				75.94	78.90	90.63	87.58	68.45	89.50	80.25	81.15	80.38	85.48	69.34	66.67	91.17	81.90
References					[18]	[27]	[28]	[29]	[8]	[30]	[31]	[32]	[33]	[34]	[35]	[36]	[37]

Table 5: HGDI scores of 24-locus MIRU-VNTR for all strains, and Beijing family strains, of TB isolated from TB patients (this study), and comparable data on Beijing family strains from previous studies.

Drug susceptibility	Number (%)	Beijing family N (%)	non-Beijing family N (%)	χ^2	P
INH-R[a]	62 (46.62)	49 (36.84)	13 (9.77)		
INH-S[b]	71 (53.38)	52 (39.10)	19 (14.29)	0.608	0.436
RIF-R	62 (46.62)	44 (33.08)	18 (13.53)		
RIF-S	71 (53.38)	57 (42.86)	14 (10.53)	1.571	0.210
EMB-R	3 (2.27)	3 (2.27)	0 (0.00)		
EMB-S	129 (97.73)	97 (73.48)	32 (24.24)	0.982	0.322
SM-R	36 (27.27)	29 (21.97)	7 (5.30)		
SM-S	96 (72.73)	71 (53.79)	25 (18.94)	0.620	0.431
CAP-R	36 (48.65)	28 (37.84)	8 (10.81)		
CAP-S	38 (51.35)	30 (40.54)	8 (10.81)	0.015	0.903
AMK-R	18 (24.32)	13 (17.57)	5 (6.76)		
AMK-S	56 (75.68)	45 (60.81)	11 (14.86)	0.532	0.466
LVFX-R	25 (33.78)	21 (28.38)	4 (5.41)		
LVFX-S	49 (66.22)	37 (50.00)	12 (16.22)	0.704	0.401
TH1321-R	7 (10.45)	5 (7.46)	2 (2.99)		
TH1321-S	60 (89.55)	48 (71.64)	12 (17.91)	0.279	0.598
MDR[c]	7 (11.48)	5 (8.20)	2 (3.28)		
XDR[d]	44 (72.13)	34 (55.74)	10 (16.39)		
AMDR[e]	10 (16.39)	9 (14.75)	1 (1.64)	1.036	0.596

[a]R: Resistance.
[b]S: Sensitive.
[c]MDR; defined as a *Mycobacterium tuberculosis* with resistance to isoniazid and rifampicin.
[d]XDR; defined as a *Mycobacterium tuberculosis* with resistance to isoniazid and rifampicin, plus resistance to a fluoroquinolone and a second-line injectable agent [38].
[e]AMDR; defined as a *Mycobacterium tuberculosis* with resistance to two or more anti-TB drugs other than MDR or XDR strains.

Table 6: Differences in drug resistance between Beijing and non- Beijing family TB strains isolated from TB patients.

Drug*	N (%)	Men N (%)	Women N (%)	χ^2	P	N (%)	Treatment history		χ^2	P	N (%)	Smoking history		χ^2	P
							Yes	No				Yes	No		
INH-R	61 (46.21)	48 (36.36)	13 (9.85)			51 (43.97)	39 (33.62)	12 (10.34)			51 (46.79)	18 (16.51)	33 (30.28)		
INH-S	71 (53.79)	58 (43.94)	13 (9.85)	0.187	0.666	65 (56.03)	11 (9.48)	54 (46.55)	41.319	**< 0.001**	58 (53.21)	34 (31.19)	24 (22.02)	5.919	**0.015**
RIF-R	61 (46.21)	50 (37.88)	11 (8.33)			50 (43.10)	38 (32.76)	12 (10.34)			50 (45.87)	20 (18.35)	30 (27.52)		
RIF-S	71 (53.79)	56 (42.42)	15 (11.36)	0.199	0.656	66 (56.90)	12 (10.34)	54 (46.55)	38.778	**< 0.001**	59 (54.13)	32 (29.36)	27 (24.77)	2.199	0.138
EMB-R	3 (2.29)	3 (2.29)	0 (0.00)			2 (1.74)	2 (1.74)	0 (0.00)			2 (1.85)	0 (0.00)	2 (1.85)		
EMB-S	128 (97.71)	102 (77.86)	26 (19.85)	0.760	0.383	113 (98.26)	47 (40.87)	66 (57.39)	2.742	0.098	106 (98.15)	52 (48.15)	54 (50.00)	1.892	0.169
SM-R	35 (26.72)	28 (21.37)	7 (5.30)			29 (25.22)	19 (16.52)	10 (8.70)			29 (26.85)	9 (8.33)	20 (18.52)		
SM-S	96 (73.28)	77 (58.78)	19 (14.50)	0.001	0.979	86 (74.78)	56 (48.70)	30 (26.09)	0.002	0.969	79 (73.15)	43 (39.81)	36 (33.33)	4.651	**0.031**
CAP-R	35 (47.95)	26 (35.62)	9 (12.33)			26 (41.94)	20 (32.26)	6 (9.68)			26 (47.27)	11 (20.00)	15 (27.27)		
CAP-S	38 (52.05)	30 (41.10)	8 (10.96)	0.222	0.638	36 (58.06)	20 (32.26)	16 (25.81)	3.011	0.083	29 (52.73)	11 (20.00)	18 (32.73)	0.109	0.741
AMK-R	17 (23.29)	14 (19.18)	3 (4.11)			14 (22.58)	10 (16.13)	4 (6.45)			14 (25.45)	7 (12.73)	7 (12.73)		
AMK-S	56 (76.71)	42 (57.53)	14 (19.18)	0.395	0.530	48 (77.42)	30 (48.39)	18 (29.03)	0.377	0.539	41 (74.55)	15 (27.27)	26 (47.27)	0.783	0.376
LVFX-R	24 (32.88)	17 (23.29)	7 (9.59)			18 (29.03)	16 (25.81)	2 (3.23)			18 (32.73)	5 (9.10)	13 (23.64)		
LVFX-S	49 (67.12)	39 (53.42)	10 (13.70)	0.692	0.402	44 (70.97)	24 (38.71)	20 (32.26)	6.581	**0.010**	37 (67.27)	17 (30.91)	20 (36.36)	1.665	0.197
TH1321-R	7 (10.61)	5 (7.58)	2 (3.03)			3 (5.45)	2 (3.64)	1 (1.82)			3 (5.45)	2 (3.64)	1 (1.82)		
TH1321-S	59 (89.39)	47 (71.21)	12 (18.18)	0.254	0.614	52 (94.55)	38 (69.09)	14 (25.45)	0.059	0.808	52 (94.55)	20 (36.36)	32 (58.18)	0.940	0.332
MDR	7 (11.67)	6 (10.00)	1 (1.67)			7 (14.00)	6 (12.00)	1 (2.00)			7 (14.00)	3 (6.00)	4 (8.00)		
XDR	43 (71.67)	34 (56.67)	9 (15.00)			33 (66.00)	27 (54.00)	6 (12.00)			33 (66.00)	12 (24.00)	21 (42.00)		
AMDR	10 (16.67)	7 (11.67)	3 (5.00)	0.648	0.723	10 (20.00)	4 (8.00)	6 (12.00)	7.556	**0.023**	10 (20.00)	4 (8.00)	6 (12.00)	0.125	0.940

*R = resistant, S = susceptible
Table 7: Association between demographic variables of TB patients, and resistance to common TB drugs of the TB strains isolated from them. P-values in bold type are statistically significant.

prevalence of the Beijing family in China as a whole was 62.24% [42].

We identified nine new spoligotypes of the Beijing family which indicates that this genotype is more diverse than previously thought. Allelic diversity varied significantly at each locus of 24-locus MIRU-VNTR which proved to be a high resolution method of genotyping *M. tuberculosis* isolates. Our results show that 24-locus MIRU-VNTR achieved a lower clustering rate and a higher HGDI than 15- and 12-loci MIRU-VNTR ($P < 0.05$). Among the loci examined, QUB11b was the most diagnostic (HGDI = 0.764 for all strains and HGDI = 0.689 for the Beijing strain). These results are consistent with those obtained from other provinces; Tibet (HGDI = 0.694), Heilongjiang (HGDI = 0.704), Shanghai (HGDI = 0.655), Beijing (HGDI = 0.646), Jiangsu (HGDI = 0.629), Inner Mongolia (HGDI = 0.702), Sichuan (HGDI = 0.783), Chongqing (HGDI = 0.729), and Tianjin (HGDI = 0.675). However, a previous study conducted during 2010 to 2011 in Jiangxi found that, although Qub11b was not the most diagnostic loci, its HGDI was high with respect to the Beijing strains [18]. Hence, Qub11b is an ideal

choice for epidemiological investigations in regions where the Beijing genotype is predominant. In addition, nine other loci; MIRU26, QUB26, Mtub04, Mtub21, ETR-E, MIRU16, Mtub39, MIRU39, and QUB4156c, had high discriminatory power in some provinces and could therefore potentially supplement Qub11b in discriminating the Beijing genotype. MIRU24 was monomorphic in 24-locus MIRU-VNTR, which confirms previous observations that this locus is phylogenetically conserved [27].

Statistical analysis indicates that patients infected with the Beijing and non-Beijing genotypes differed significantly in age composition ($P < 0.05$). There was, however, no association between the prevalence of the Beijing genotype and gender, treatment or smoking history ($P > 0.05$). The relationship between the Beijing genotype and demographic variables is still poorly understood; demographic factors may be responsible for the predominance of Beijing genotype strains through co-evolution between host and pathogen. These results may, however, be biased by the relatively small sample size of non-Beijing genotype compared to that of the Beijing genotype and there may also be other factors that contribute to the predominance of the Beijing genotype. Further investigation of the role of demographic factors in the population structure of *M. tuberculosis* in China is required [35,43].

Drug resistance is a major public health problem that impedes TB treatment and control. According to the 2015 Global tuberculosis report, 3.3% of new and 20% of previously treated, TB patients were estimated to have had MDR-TB in 2014. In our study, 56.39% (75 isolates) were resistant to at least one drug, and among these, 5.26% (7 isolates) were MDR, 33.08% (44 isolates) were XDR and 7.52% (10 isolates) were AMDR, which was significantly different from the previous study during 2010 to 2011 (the proportion of MDR and XDR were 19.8% and 10.6% respectively) [18]. This may because the samples collected in our study were from clinical isolates which have been treated. And the epidemiological data showed that some of the TB patients were repeatedly treated during those three years. Hence, the proportion of XDR was significantly increased. Although we did not find a significant difference in drug resistance between the Beijing and non-Beijing family strains, this may have been because of the relatively small sample size available. Other factors may, however, have contributed to this result, including the different proportions of Beijing family subgroups. Mokrousov et al. (2006) demonstrated that different sublineages of the Beijing family may differ in their mechanisms of adaptation to the selective pressure imposed by drug treatments [44]. Further research on this in TB isolates from Jiangxi is required. Consistent with a previous study in Jiangsu province, our results show that treatment history was significantly associated with resistance to INH, RIF and LVFX ($P < 0.05$) [45]. And the risk factor analyzes suggested that treatment was a risk factor to INH, RIF and LVFX resistances ($OR > 1$). Furthermore, more frequent drug treatment was also associated with the development of multiple drug resistance ($P < 0.05$). These results suggest that drug treatments should be minimized in order to reduce the development of drug resistance and MDR strains of TB.

Interestingly, we found evidence of a negative association between smoking and resistance to INH and SM ($P < 0.05$). Many studies have demonstrated that smokers have an increased risk of clinical TB presentation, duration of sputum and culture positivity, unfavorable treatment outcomes, and risk of relapse [46-48]. Pai et al. found considerable evidence that smoking was associated with TB [49]. There has, however, been little research on the association between smoking and drug-resistant strains of TB. An important systematic review and meta-analysis conducted in 2007 found strong evidence of an association between smoking and TB, moderate evidence of an association between smoking and the recurrence of TB, and insufficient evidence to support an association between smoking and drug-resistance TB [50]. Our results show a negative association between smoking and drug resistance to INH and SM. This suggests that smoking was a protect factor to INH and SM resistance. However, our study was correlational, rather than experimental. Further research is required to confirm the existence of a relationship between smoking and drug-resistance in TB.

Conclusion

This is a systematic investigation of the molecular epidemiology of *M. tuberculosis* isolated from Jiangxi province, China. The Beijing family is the most prevalent TB strain. A combination of spoligotyping and 24-locus MIRU-VNTR proved useful for the epidemiological analysis of TB in this area. More than half the *M. tuberculosis* isolates collected were resistant to anti-TB drugs, and the majority of these were resistant to more than one drug. There was, however, no relationship between genetic diversity and drug resistance in *M. tuberculosis*. Moreover, our results suggest that treatment history can lead to the development of drug resistance ($P < 0.05$), which supports the more moderate use of medication in the treatment of TB.

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