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Research Article

Multipleloci Variable-number Tandem Repeat Typing of Clinical *Mycobacterium tuberculosis* Isolates from Zunyi, Guizhou Province of China

Abstract

Introduction: Due to the emergence of multidrug/extensively drug-resistant TB and the lack of new anti-TB drugs, tracing the infectious source and monitoring the transmission of drug-resistant TB strains have become critically important.

Methods: To determine which VNTR locus set is more suitable for use in Zunyi, China, we used 19 VNTR loci including 4 recommended by the China CDC, 4 recommended a group of scientists from Europe and USA, and 11 recommended by both for molecular typing of 100 clinical *M. tuberculosis* isolates from Zunyi, Guizhou Province of China. PCR amplified DNA fragments were analyzed by comparing their sizes on the gel images with those amplified from the reference *M. tuberculosis* H37Rv strain at each of the 19 corresponding VNTR loci to determine copy numbers of each VNTR. The cluster analysis was carried out by using the Bionumerica 5.0 software.

Results: High polymorphism in loci MIRU26, Mtub4, Mtub21, QUB11B, and QUB26, and low polymorphism in loci ETRC and MIRU23 were detected in 100 *M. tuberculosis* strains isolated from Zunyi, Guizhou Province of China. The Hunter-Gaston index values for 15-VNTR$_{China}$ and 15-VNTR$_{Internation}$ are 0.9992 and 1 respectively.

Conclusion: Results from this study indicate that the 15-VNTR$_{Internation}$ has the higher discrimination power than the 15-VNTR$_{China}$ for molecular genotyping of clinical *M. tuberculosis* isolates from Zunyi, and is an appropriate tool for molecular epidemiological studies of *M. tuberculosis* in Zunyi, Guizhou Province of China.

Introduction

Tuberculosis (TB) is one of the leading infectious disease killers in the world which caused about 1.5 million deaths worldwide in 2013 [1]. China had the second largest number of TB cases among 22 high TB-burden countries in the world with the estimated TB incidence rate of 94 per 100,000 people [2]. To completely eliminate TB in the world, the effective prevention, control and treatment of TB are considered to be the best measures. However, due to the emergence of multidrug/extensively drug-resistant TB (MDR/XDR-TB) and the lack of new anti-TB drugs as well as effective vaccines against TB, tracing the infectious source and monitoring the transmission of drug-resistant TB strains have become critically important.

During last fifteen years, molecular biology methods such as PCR-based spoligotyping, IS6110 restriction fragment length polymorphism (RFLP) and multilocus variable-number tandem repeat analysis (MLVA) have been widely used in epidemiologic studies of TB [3,4]. MLVA is a PCR-based method for genotyping of Mycobacterium tuberculosis complex, and its advantages include rapid, ease of use, low cost, good reproducibility, and convenient for data to be digitalized, analyzed and shared among different laboratories. The discriminatory power of MLVA is mainly determined by the selection of variable-number tandem repeat (VNTR) loci. MLVA has been used for molecular typing of *M. tuberculosis* isolates from 38 countries [5], and different provinces of China including Tibet [6], Jiangsu [7], Sichuan [8], Gansu [9], Shanghai [10], and Jiangxi [11]. However, MLVA has not been used for molecular typing of *M. tuberculosis* isolates from Zunyi, Guizhou Province of China. In order to determine which VNTR locus set is more suitable for genotyping of *M. tuberculosis* isolates in Zunyi, we used 19 VNTR loci including 4 recommended by the China CDC [12], 4 recommended a group of scientists from Europe and USA [5], and 11 recommended by both for molecular typing of 100 clinical *M. tuberculosis* isolates consecutively collected between January and December 2013 from patients with pulmonary tuberculosis at the Affiliated Hospital of Zunyi Medical College, Guizhou Province. We also compared our results with those from different provinces of China [6-11].

Materials and Methods

Study setting

The Medical Ethics Committee at Affiliated Hospital of Zunyi Medical College approved this study. Sputum samples from 161 patients who were diagnosed as active pulmonary tuberculosis

by acid-fast bacilli staining were consecutively collected between January and December 2013 at the Affiliated Hospital of Zunyi Medical College, Guizhou Province. Out of them, sputum samples from 100 TB patients were culture positive, while the other 61 sputum samples were culture negative. All patients were screened for human immunodeficiency virus (HIV) infection and none of them was positive.

M. tuberculosis strains

Sputum specimens were inoculated on Löwenstein-Jensen slants at the Laboratory of Respiratory Medicine, Affiliated Hospital of Zunyi Medical College, Guizhou Province of China, between January and December of 2013. Only one clinical isolate of *M. tuberculosis* was collected from each patient, and a total of 100 clinical isolates were determined to be *M. tuberculosis* and used for this study. The standard *M. tuberculosis* strain $H37R_v$ and *Mycobacterium bovis* BCG were obtained from the China CDC.

Reagents

Taq DNA polymerase, 100 bp DNA Ladder, dNTP, etherdium bromide were purchased from Sino-American Biotechnology Co., Ltd (Henan, China); Biodyne C membrane was purchased from Pall Corporation (Port Washington, NY, USA); streptavidin was purchased from Sigma-Aldrich (St. Louis, MO, USA); and Spoligotyping Kit was purchased from Isogen Bioscience BV (Maarsen, Netherlands).

Preparation of mycobacterial DNA

All clinical isolates of *M. tuberculosis* were grown on fresh Löwenstein-Jensen (L-J) slants at 37°C for 4 to 6 weeks. One to two loops of *M. tuberculosis* colonies were removed from L-J slants, suspended in 400 µl of Tris-EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH8.3) in a 1.5 ml Eppendorf tube by vortexing, and boiled for 30 min. The mixture was centrifuged at 12,000 *rpm* for 3 min, and the supernatant was transferred to a new Eppendorf tube and stored at -20°C until use.

VNTR genotyping

Primer sequences, expected sizes of PCR products and copy numbers in the $H37R_v$ genome for 19-loci VNTRs were listed in Table 1. Fifteen of the 19-loci VNTRs ($15\text{-}VNTR_{China}$) were selected by Institute of Infectious Disease Prevention and Control of the China CDC [12] for genetic fingerprinting of clinical *M. tuberculosis* isolates. These $15\text{-}VNTR_s$ have the same melting temperature (Tm), can be handled easily, and give consistent results which make it easy to conduct the comparative analysis. Eleven of these $15\text{-}VNTR_s$ were originally proposed by a group of scientists from the Europe and USA as the new standard for routine epidemiological discrimination of *M. tuberculosis* [5], and the rest four loci ($QUB4156_c$, Mtub04, QUB-11b and QUB-26) were selected from the new standard 15-locus system ($15\text{-}VNTR_{International}$) [5].

PCR amplification was performed in a final volume of 16 µl containing 1.6 µl of 10×PCR reaction buffer; 50 pmol of $MgCl_2$; 10 pmol of $dNTP_s$; 20 pmol of forward and reverse primers; 1U of *Taq* DNA polymerase; and 3 µl of DNA template (20-100ng/µl). Amplification was carried out in a thermal cycler (DNA Engine, BIO-RAD, USA) by using the following parameters: an initial denaturation of 5 min at 94°C followed by 35 cycles consisting of denaturation at 94°C for 30s, primer annealing at 62°C for 30s, and extension at 70°C for 45s; and a final extension step at 72°C for 10min. Genomic DNA purified from the standard *M. tuberculosis* strain $H37R_v$ was used as the positive control, and double distilled water as the negative control. PCR products were applied to 2% of agarose gels in the TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH7.5) containing 0.5 µg/ml of ethidium bromide for electrophoresis. The PCR-amplified DNA products were visualized and photographed under the UV light. The 100 bp DNA ladders were used as DNA markers.

PCR products amplified from 100 *M. tuberculosis* strains were analyzed by comparing PCR product sizes on the gel images with those amplified from the reference $H37R_v$ strain at each of the 19 corresponding VNTR loci (Table 1) to determine copy numbers of each VNTR. By selection of different combinations of 19 VNTR loci, $15\text{-}VNTR_{China}$ and $15\text{-}VNTR_{International}$ (Table 1), the obtained results were further analyzed by using the Bionumerics (Version 5.0, Applied Maths, St. Marten-Latem, Belgium) software, the Dice Similarity Coefficient was calculated with UPGMA, and genotypes for 100 *M. tuberculosis* strains were classified according to their differences in Cluster Cutoff values.

Allelic diversity analysis

The following formula [13] was used to calculate the VNTR allelic diversity *(h)*:

$$h = 1 - \sum x_i^2 \left[n/(n-1) \right]$$

Where x_i represents the frequency of the allele *i* in VNTR loci, and *n* is the total number of *M. tuberculosis* isolates.

The calculation of the discriminatory power was done by using the Hunter-Gaston index (HGI) for VNTR based on the following resolution formula [14]:

$$HGI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j \ (n_j - 1)$$

Where *N* represents the total number of *M. tuberculosis* strains, *S* is the total number of genotypes by different genotyping methods, and *nj* is the strain number belonging to the genotype *J*. The HGI value was used to evaluate two different VNTR locus combinations for molecular genotyping of *M. tuberculosis* strains from Zunyi, Guizhou Province of China.

Results

Study population

The Medical Ethics Committee at Affiliated Hospital of Zunyi Medical College approved this study. Among 100 patients with active pulmonary tuberculosis, 60 were male and 40 were female; 72 were new and 28 were previously treated cases; 60 were between ages of 20 and 50, 34 were over 50 and 6 were below 20 years of age.

Quality control and reproducibility of results

A negative control (deionized water instead of purified DNA templates) and the positive control (purified DNA from the reference strain $H37R_v$) were included for every PCR. Experimental results

Table 1: Primer sequence, size of PCR product and copy number of each VNTR locus in the $H37R_v$ genome.

VNTR locus	PCR primer sequence (5'-3')	Repeat unit length (bp)	Copies in $H37R_v$	Size of PCR product (bp)
ETR-Aa,b	F: ATTTCGATCGGGATGTTGAT	75	3	397
	R: TCGGTCCCATCACCTTCTTA			
ETR-Bb	F: GCGAACACCAGGACAGCATCATG	57	3	292
	R: GGCATGCCGGTGATCGAGTGG			
ETR-Ca,b	F: GACTTCAATGCGTTGTTGGA	58	4	346
	R: GTCTTGACCTCCACGAGTGC			
ETR-Da,b	F: CAGGTCACAACGAGAGGAAGAGC	77	3	310
	R: GCGGATCGGCCAGCGACTCCTC			
ETR-Ea,b	F: ACTGATTGGCTTCATACGGCTTTA	53	3	651
	R: GTGCCGACGTGGTCTTGAT			
MIRU10a,b	F: GTTCTTGACCAACTGCAGTCGTCC	53	3	643
	R: GCCACCTTGGTGATCAGCTACCT			
MIRU16a,b	F: TCGGTGATCGGGTCCAGTCCAAGTA	53	2	671
	R: CCCGTCGTGCAGCCCTGGTAC			
MIRU23b	F: CAGCGAAACGAACTGTGCTATCAC	53	6	873
	R: CGTGTCCGAGCAGAAAAGGGTAT			
MIRU26a,b	F: CCCGCCTTCGAAACGTCGCT	51	3	614
	R: TGGACATAGGCGACCAGGCGAATA			
MIRU27b	F: TCGAAAGCCTCTGCGTGCCAGTAA	53	3	657
	R: GCGATGTGAGCGTGCCACTCAA			
MIRU39b	F: CGCATCGACAAACTGGAGCCAAAC	53	2	646
	R: CGGAAACGTCTACGCCCCACACAT			
MIRU40a, b	F: GGGTTGCTGGATGACAACGTGT	54	1	407
	R: GGGTGATCTCGGCGAAATCAGATA			
Mtub04a	F: GTCCAGGTTGCAAGAGATGG	51	3	250
	R: GGCATCCTCAACAACGGTAG			
Mtub21a,b	F: AGATCCCAGTTGTCGTCGTC	57	2	206
	R: CAACATCGCCTGGTTCTGTA			
Mtub30a,b	F: AGTCACCTTTCCTACCACTCGTAAC	58	2	319
	R: ATTAGTAGGGCACTAGCACCTCAAG			
Mtub39a,b	F: AATCACGGTAACTTGGGTTGTTT	58	6	515
	R: GATGCATGTTCGACCCGTAG			
QUB-11ba	F CGTAAGGGGGATGCGGGAAATAGG	69	5	412
	R: CGAAGTGAATGGTGGCAT			
QUB-26a	F: GGCCAGGTCCTTCCCGAT	111	5	708
	R: AACGCTCAGCTGTCGGAT			
QUB4156ca	F: TGGTCGCTACGCATCGTGTCGGCCCGT	59	2	224
	R: TACCACCCGGGCAGTTTAC			

[a]15-VNTR-International; [b]15-VNTR-China; VNTR, variable-number tandem repeat.

showed that non-specific DNA bands were not seen in the negative lanes on gel images, and amplification products from the positive control yielded DNA bands with the expected size for each VNTR locus. To test whether experimental results could be reproduced, 19 VNTR loci were amplified for three times separately from 30 randomly selected *M. tuberculosis* strains, and the same-size PCR products were observed for each of the three experiments which demonstrated the reproducibility of our experimental results.

Multipleloci VNTR analysis of M. tuberculosis strains

A total of 100 *M. tuberculosis* clinical strains from Zunyi, Guizhou Province of China, were analyzed by comparing PCR product sizes on gel images with those amplified from the reference $H37R_v$ strain. Copy numbers for each of the 19 corresponding VNTR loci were further analyzed by using the Bio Numerics (Version 5.0) database software, the Dice Similarity Coefficient was calculated with the UPGMA, and genotypes were classified according to their differences in Cluster Cutoff values. Results showed that different strains expressed variable polymorphisms at each of the 19 VNTR loci, and polymorphisms among these VNTR loci were quite different (Tables 2,3). The loci MIRU26 and QUB11b demonstrated the highest diversity (h=0.838 and h=0.824), whereas loci MIRU23 and ETRC showed the lowest diversity (h=0.125 and h=0.210 respectively). Polymorphisms in other VNTR loci were listed in Tables 2,3.

Table 2: Allele diversity (h) and copy numbers at 19 selected VNTR loci for 100 *M. tuberculosis* isolates from Zunyi, Guizhou province of China.

VNTR locus	Allele diversity (h)	No. of groups	No. of isolates with different copy numbers of tendem repeat unit(s)									
			0	1	2	3	4	5	6	7	8	9
MIRU26a,b	0.838	10	2	4	2	3	2	17	15	21	21	13
QUB11Ba	0.824	8	3		17	15	15	22	22	5	1	
Mtub21a,b	0.737	6		10	4	37	26	21				2
ETREa,b	0.728	8		1	1	35	30	23	4	4	2	
QUB26a	0.713	8	1		5	1	5	4	24	16	44	
Mtub4a	0.697	5			1	33	17	39	10			
MIRU40a,b	0.669	7		3	16	51	19	5	5		1	
MIRU10a,b	0.640	6		9	44	39	4	2	2			
ETRAa,b	0.630	6	1	1	16	50	30	2				
MIRU16a,b	0.586	6	4	1	60	13	17	5				
ETRDa,b	0.552	7		1	28	60	1	7	2		1	
QUB4156ca	0.521	5		6	65	6	21	2				
MIRU39b	0.517	4		1	57	39		3				
Mtub30a,b	0.504	3			48	1		51				
Mtub39a,b	0.469	7	1	1	11	4	71	5	7			
ETRBb	0.469	3		34	64	2						
MIRU27b	0.409	2			15	85						
ETRCa,b	0.210	3			5	7	88					
MIRU23b	0.125	5		2	1		2	93	2			

[a]15-VNTR-International; [b]15-VNTR-China; VNTR, variable-number tandem repeat.

Table 3: Comparison of allele polymorphisms (h) and Hunter-Gaston discrimination index (HGDI) of 19 selected VNTR loci for *M. tuberculosis* isolates from different cities and provinces of China.

VNTR locus	HGDI for M. tb isolates from different provinces of China						
	Guizhou	Tibet	Jiangsu	Sichuan	Gansu	Shanghai	Jiangxi
ETRA	0.630	0.169	0.313	0.514	0.351	0.397	0.386
ETRB	0.469	0.109	0.096	0.300	0.170	/	0.221
ETRC	0.210	0.110	0.090	0.168	0.051	0.111	0.157
ETRD	0.552	0.097	0.592	0.328	0.116	0.514	0.270
ETRE	0.728	0.663	0.695	0.558	0.511	0.500	0.474
MIRU10	0.640	0.193	0.446	0.545	0.339	0.488	0.501
MIRU16	0.586	0.222	0.348	0.384	0.403	0.248	0.300
MIRU23	0.125	0.031	0.268	0.077	0.079	/	0.179
MIRU26	0.838	0.482	0.683	0.764	0.627	0.632	0.580
MIRU27	0.409	0.078	0.243	0.413	0.114	/	0.222
MIRU39	0.517	0.243	0.390	0.503	0.242	0.318	0.410
MIRU40	0.669	0.251	0.454	0.572	0.335	0.417	0.407
Mtub04	0.697	0.278	0.467	/	/	0.514	/
Mtub21	0.737	0.539	0.660	0.772	0.694	0.658	0.655
Mtub30	0.504	0.151	0.365	0.479	0.265	0.341	0.309
Mtub39	0.469	0.257	/	0.591	0.253	0.407	0.300
QUB11b	0.824	0.75	0.717	/	/	0.771	/
QUB26	0.713	0.583	0.646	/	/	0.627	/
QUB4156c	0.521	/	/	/	/	0.385	/

VNTR, variable-number tandem repeat; and /, no data; HGDI, Hunter-Gaston discrimination index.

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Analysis of *M. tuberculosis* strains with 15-VNTR$_{China}$ and 15-VNTR$_{International}$

One hundred *M. tuberculosis* strains from Zunyi were also analyzed by using two different combinations of VNTR loci, 15-VNTR$_{China}$ and 15-VNTR$_{International}$. Results showed that 100 clinical *M. tuberculosis* strains from Zunyi could be classified into 97 unique genotypes by using the combination of 15-VNTR$_{China}$, 95 of them had unique genotypes, and the rest five strains were clustered into two groups with the cluster rate of 5% and the Hunter-Gaston index (HGI) of 0.999. Using the combination of 15-VNTR$_{International}$, we found that 100 *M. tuberculosis* strains from Zunyi belonged to 100 unique genotypes with the Hunter-Gaston index (HGI) of 1, indicating that 15-VNTR$_{Internation}$ had the higher discrimination power than 15-VNTR$_{China}$ for molecular genotyping of *M. tuberculosis* clinical strains from Zunyi.

Discussion

A total of 100 clinical *M. tuberculosis* isolates collected from Zunyi, Guizhou Province of China, between January and December 2013 were genotyped by the multilocus variable-number tandem repeat analysis (MLVA). Results showed that polymorphisms at each of the 19 VNTR loci were observed for different strains, and polymorphisms among these VNTR loci were quite different (Tables 2,3). The loci MIRU26 and QUB11b demonstrated the highest diversity (h=0.838 and h=0.824), whereas loci MIRU23 and ETRC showed the lowest diversity (h=0.125 and h=0.210 respectively). Polymorphisms in other VNTR loci were listed in Tables 2,3.

Sola et al. divided the polymorphisms of alleles at different loci into 3 groups based on the HGI values: high HGI (h>0.6), moderate HGI (0.3≤h≤0.6) and low HGI (h<0.3) [13]. The molecular genotyping results using the 19 loci VNTR indicate that different loci show varying HGI values and polymorphisms. High polymorphisms (h>0.6) in loci MIRU26, Mtub4, Mtub21, QUB11B and QUB26, and low polymorphisms (h<0.3) in loci ETRC and MIRU23 have been detected in 100 *M. tuberculosis* clinical strains isolated from Zunyi, Guizhou Province of China. We examined the relationship between h and HGI values, and found out that higher h values corresponded to higher HGI values. Consequently, the genetic variation at a particular locus determines its HGI value to a certain extent. Meanwhile, there are distinct regional disparities of the HGI values at different loci. However, the HGI values for *M. tuberculosis* strains from Zunyi area of Guizhou Province exhibited a great similarity to those from Sichuan Province (Tables 2,3), indicating that the HGI value at a particular locus is related to the evolution rate of the locus and the geographical location where the isolate is collected.

In view of variant polymorphisms in different loci combinations for *M. tuberculosis* isolates from different countries, different areas of the same country and different strains from the same region, it is essential to select an appropriate set of VNTR$_s$ suitable for molecular genotyping of *M. tuberculosis* strains isolated from Zunyi, Guizhou Province of China. The 15-VNTR$_{International}$ (including 6 MIRU loci) is recommended as the loci combination for molecular epidemiological investigations [5]. One hundred of *M. tuberculosis* clinical isolates from Zunyi can be divided into 100 unique genotypes using the 15-VNTR$_{International}$ combination, and the HGI value is 1. Dong et al utilized 15-VNTR$_{International}$ to genotype *M. tuberculosis* isolates from Mianyang area of Sichuan Province [8], and the HGI result is consistent with that of Zunyi area. The 15-VNTR$_{China}$ (including 9 MIRU loci) is selected by the National TB Key Laboratory of China CDC as the standard combination for *M. tuberculosis* MLVA analysis in China [12]. Using the 15-VNTR$_{China}$, one hundred of *M. tuberculosis* clinical isolates from Zunyi can be divided into 2 clusters and 97 genotypes with the cluster rate of 5%. The HGI value for isolates from Zunyi is 0.9992, which is higher than that derived from Anhui (0.913) [9] and Sichuan (0.994) [10] provinces. It seems that the same combination of MLVA could display different polymorphisms for *M. tuberculosis* isolates from different geological regions, and therefore, the selection of an appropriate MLVA loci combination is necessary for molecular genotyping of *M. tuberculosis* isolates from a particular region.

In conclusion, results from this study indicate that 15-VNTR$_{Internation}$ has the higher discrimination power than the 15-VNTR$_{China}$ for molecular genotyping of *M. tuberculosis* clinical strains from Zunyi, and is an appropriate tool for future epidemiologic studies of *M. tuberculosis* isolates in Zunyi, Guizhou Province of China.

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