Letter to the Editor

Molecular Characterization and Drug-resistance of *Mycobacterium tuberculosis* Strains in Xuzhou, China*

PANG Hui[1,2,3,‡], TONG Jing[4], LIU Hai Can[2,5], DU Yang Guang[4], ZHAO Xiu Qin[2,5], JIANG Yi[2,5], WU Xiao Cui[2,5], YANG Jin Chuan[4,#], and WAN Kang Lin[2,5,#]

To understand the genetic diversity and drug resistance status of *Mycobacterium tuberculosis* (*M. tuberculosis*) circulating in Xuzhou of China, the spacer-oligonucleotide typing (Spoligotyping) and multi-loci VNTRs (variable number tandem repeats) analysis (MLVA) were utilized for the genotyping of the isolates. Drug susceptibility test (DST) was performed by the proportion method on the Lowenstein-Jensen (L-J) medium using isoniazid, rifampicin, ethambutol, and streptomycin. By Spoligotyping, 287 *M. tuberculosis* isolates were differentiated into 14 clusters. Then with 15-loci MLVA, these strains could be divided into 32 clusters, 228 genotypes. Of 15 VNTRs, 6 loci had the highly discriminatory powers, 6 loci presented moderate discrimination and 3 loci demonstrated less polymorphism. The DST results showed that 46 strains were resistant to at least one first-line anti-tuberculosis agent. There was a difference in the isoniazid resistance between Beijing and non-Beijing genotype strains. We concluded that the combination of Spoligotyping and 15 VNTR loci as the genotyping in our study was applicable for this region, the drug resistant isolates were identified, and the Beijing family was the most prevalent genotype in the rural counties of Xuzhou.

Tuberculosis (TB) remains a major cause of global public health problem, mainly in the developing countries[1]. Of 22 high TB burden countries, China ranks the second, just behind India, and reported 0.86 million new cases in 2012[1]. In China, approximately 80% of TB cases are in rural areas[2]. Xuzhou is an important gateway city and a new international energy base located in the eastern China. A great many TB cases in our research were obtained from the counties of Xuzhou. Therefore, there is an urgent need for investigating molecular epidemiology and drug resistance of TB in this region in order to provide preventive and therapeutic strategies.

The genotyping of *Mycobacterium tuberculosis* (*M. tuberculosis*) is important for TB control because it allows the detection of suspected outbreaks, the tracing of transmission chains and identifying secondary infections[3]. The genetic markers for genotyping of *M. tuberculosis* should have the genetic stability and the diversity of individuals.

Among the genotyping tools, the spacer-oligonucleotide typing (Spoligotyping) is an ideal typing method based on PCR[4] and is the golden standard to identify strains belonging to the Beijing family[4]. The multi-loci VNTRs (variable number tandem repeats) analysis (MLVA) is another newly good method which is also a kind of DNA fingerprint technique based on PCR[5]. The genomic DNA of *M. tuberculosis* has a number of independent VNTRs. VNTRs have high polymorphism, which can determine the number of repeated mycobacterial interspersed repetitive units (MIRU). The results are shown by the digital format pattern and can be facilitated by interlaboratory comparison all over the world. Different laboratories can search for the fitful loci for the genotyping of *M. tuberculosis* from different areas. In this research, we collected clinical *M. tuberculosis* isolates for investigating the genetic diversity, genotyping characteristics and drug resistances of *M. tuberculosis* circulating in Xuzhou of China, by Spoligotyping and MLVA with 15 VNTR

doi: 10.3967/bes2014.136

*This work was funded by the projects 2013ZX10003002-001 of Chinese National Key Program of Mega Infectious Disease of the National 12th Five-Year Plan and the Science and Technology Innovation Team Support project CX201412 of Changzhi Medical College.

1. Department of Immunology, Changzhi Medical College, Changzhi 046000, Shanxi, China; 2. State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 3. Department of Immunology, Xiangya School of Medicine, Central south University, Changsha 410078, Hunan, China; 4. Xuzhou Municipal Centre for Disease Control and Prevention, Xuzhou 221006, Jiangsu, China; 5. Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310000, Zhejiang, China

loci[6].

A total of 287 clinical *M. tuberculosis* strains in our study were isolated from TB patients with sputum smear positive from January of 2011 to December of 2012 in the seven counties of Xuzhou, including 2 from Jiawang, 14 from Tongshan, 35 from Xinyi, 47 from Peixian, 47 from Fengxian, 64 from Suining, and 78 from Pizhou. The average age of the patients was 51, and 80.14% (230/287) were males. The reference strain was H37Rv.

The genomic DNA was extracted from the *M. tuberculosis* cultured on Lowenstein-Jensen (L-J) medium using the CTAB (cetyltrimethylammonium bromide)-NaCl method as described previously[7]. Spoligotyping was performed according to the standard protocol by Kamerbeek et al.[4]. And the Spoligotypes were received by binary format with the SpolDB4.0 database (http://www.pasteur-guadeloupe.fr:8081/SITVITD). According to the former investigation[6], 15 VNTR loci were selected for MLVA typing for *M. tuberculosis* genetic diversity study in China.Every experimental strain was analyzed by replication numbers of every VNTR locus compared with H37Rv. The results were analyzed by Bionumerics 5.0 software. Categorical and unweighted pair group methods using arithmetic averages (UPGMA) index were used in order to be adapted to clustering analysis. Genotyping was performed by the difference of the cluster cutoff value. The Hunter-Gaston discriminatory index (HGDI) was used to estimate the allelic diversity of the VNTR locus and the cumulative HGDI can evaluate the discriminatory power of the VNTR loci. The clustering rate was calculated by the formula[8]. The same cluster was defined with the identical or highly similar DNA fingerprint of a group of *M. tuberculosis*.

As for the drug susceptibility test (DST), the L-J medium was impregnated with isoniazid (INH), rifampicin (RFP), ethambutol (EMB), and streptomycin (SM), in conformity with the proportional technique as recommended by World Health Organization (WHO) and Clinical and Laboratory Standards Institute (CLSI). The concentrations of the drugs INH, RFP, EMB, and SM in the media were 0.2 μg/mL, 40 μg/mL, 2 μg/mL, and 4 μg/mL, respectively. If the microorganism growing on the particular drug medium was ≥1% or <1% compared to that growing on the control culture, the strain was detected as resistant or sensitive individually. χ^2 test and Fisher exact probability were used for analyzing two or more samples within SPSS 17.0 software. The differences were based on α=0.05.

The results of Spoligotyping clustering analysis exhibited that 287 strains could be differentiated into 14 clusters, in which 230 (80.14%) were clustered into Beijing family including typical Beijing genotype (227, 79.09%) and Beijing-like genotype (3, 1.05%), 30 (10.45%) were T family, 4 (1.39%) were U genotype, 3 (1.05%) were MANU2 genotype, 1 (0.35%) was H3, and 19 strains were not identified in the SpolDB4.0 database and referred to 13 'new' genotypes (Table 1). The cumulative Hunter-Gaston discriminatory index (HGDI) was 29.60% and the clustering rate was 89.20%. The results supported the view that Beijing strains were predominant in different areas of China and other countries all over the world[9-10]. However, the spoligotyping method failed to effectively distinguish genetic diversity among Beijing family strains.

Then, the isolates were genotyped by the MLVA with 15 VNTR-loci, and the results demonstrated that 287 isolates were divided into 32 clusters and 228 genotypes including 196 unique patterns. The clustered isolates were 92 and the clustering rate was 20.91%, which was assumed to be the ratio of clustered strains in a population and the level of active transmission as well. There were 2 to 6 strains in each cluster. The suitable loci were selected principally according to the population structure of *M. tuberculosis* in the investigated region. Each of 15-loci showed different discriminatory power. The HGDI varied significantly from 0.761 for MIRU26 to 0.120 for ETRC. The highly discriminatory powers loci were MIRU26, Mtub21, ETRD, MIRU10, Mtub30, and ETRE. MIRU27, MIRU16, MIRU39, ETRA, Mtub39, and MIRU23 demonstrated moderate discriminatory power, and 3 loci (MIRU40, ETRB, and ETRC) were found to be less polymorphic. MIRU26 locus showed the highest polymorphism in Xuzhou, but this locus was not the best in Tibet, Heilongjiang, Taipei, and South Korea[9-10]. ETRD, MIRU10, Mtub30, and ETRE also revealed higher diversity than in other zones[9]. Mtub21 was the similarly high discriminatory in other areas, such as in Inner Mongolia and Heilongjiang. ETRB and ETRC were also less polymorphous in Heilongjiang, Tibet and Inner Mongolia. The cumulative HGDI was 99.73%. From the previous study, we found that the cumulative HGDI of 15- and the 16-locus set were the same as 0.9977, and that of the 10- and 11-locus were equal to 0.9950. All VNTR-loci repeats of the isolates compared with H37Rv were shown in Table 2.

In comparison with the genotyping results of

287 isolates with Spoligotyping and 15 VNTR-loci MLVA, there were some distinctions between them. Although Spoligotyping can be considered the gold standard for identifying Beijing family strains, it is uninformative to Beijing strains in virtue of the genetic homogeneity of this family. MLVA is a high throughput technique which can be adapted to distinguish Beijing genotyping isolates. The discriminatory powers of 15 VNTR loci among Beijing and non-Beijing strains were 99.62% and 99.44% respectively. The 230 Beijing family strains could be divided into 180 genotypes, 28 clusters by the MLVA method, but it was displayed that 5 non-Beijing family bacteria were clustered into the Beijing typing. The reason for 5 non-Beijing family bacteria clustered into the Beijing typing might be the mixed infection in the experimental strains. Obviously, the discriminatory power of the MLVA with 15 VNTR-loci method was much higher than that of Spoligotyping for analyzing Beijing genotypes' strains.

The DST results showed that 16.03% (46/287) were resistant to at least one of the four first-line drugs. The proportions of single-drug resistance were 0.70% (2/287) for RFP, 4.88% (14/287) for INH, and 3.48% (10/287) for SM. Among all isolates, 9 (3.14%) isolates were multi-drug resistant (MDR) strains including 2 (0.70%) strains resistant to all four first-line drugs. Isoniazid resistance rate was significantly different between Beijing genotype and non-Beijing genotype strains (Table 3). The MDR rates in Heilongjiang, Liaoning, Henan, Inner Mongolia, and Shanghai were 7.2%, 10.4%, 7.8%, 7.0%, and 3.0%, respectively. Those data were lower than the average level all over China. It might be

Table 1. Spoligotypes of *M. tuberculosis* Isolates (*n*=287)

Spoligotypes	SIT[1]	Genus[2]	*N*[3] (%)
	1	BEIJING	227 (79.09)
	269	BEIJING-LIKE	3 (1.05)
	334	T1	3 (1.05)
	804	T1	2 (0.70)
	53	T1	8 (2.79)
	196	T1	1 (0.35)
	1112	T1	2 (0.70)
	78	T1-T2	1 (0.35)
	52	T2	4 (1.39)
	118	T2	2 (0.70)
	875	T2	6 (2.09)
	135	T2	1 (0.35)
	523	U	1 (0.35)
	124	U	1 (0.35)
	246	U	1 (0.35)
	1311	U	1 (0.35)
	54	MANU2	3 (1.05)
	390	H3	1 (0.35)
	-	NEW	2 (0.70)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	3 (1.05)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	2 (0.70)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	1 (0.35)
	-	NEW	3 (1.05)

Note. [1]: SIT: spoligotype international type from the Spoldb4.0 database; [2]: Genus: spoligotype genus in the Spoldb4.0; [3]: N: the number of the strains with the same SIT.

attributed to excellent prevention and control work which resulted in reduction of the spread of drug resistant tuberculosis in Xuzhou. Among the single drug resistance, the resistant rate of *M. tuberculosis* to INH is the highest, and the following is to SM, RFP. The single EMB-resistance was not found in this study. It was suggested that clinicians could use EMB replaced INH and SM. Thirty five drug-resistant strains were typical Beijing genotype among all the resistant isolates. It was demonstrated that Beijing family strains could be more virulent and associated with drug resistance. It was displayed that the resistant isolates were mainly separated from the counties of Pizhou and Suining within Xuzhou. And the MDR strains were largely from Suining. Consequently, it is necessary to strengthen the current TB control strategies and surveillance programs in these areas.

Table 2. The Repeat Numbers of VNTR-Loci for 287 *M. tuberculosis* Clinical Strains

VNTR Loci	Size of Repeat in H37Rv (bp)	No. of Repeats in H37Rv	Repeat Numbers of VNTR-loci in the Clinical Isolates											
			1	2	2.5	3	4	5	6	7	8	9	10	11
ETRA	75	3	-	30	-	54	195	2	-	-	-	6	-	-
ETRB	57	3	30	254	-	3	-	-	-	-	-	-	-	-
ETRC	58	4	-	2	-	11	270	2	2	-	-	-	-	-
ETRD	77	3	7	96	101	57	17	8	-	1	-	-	-	-
ETRE	53	3	-	3	-	19	35	158	68	4	-	-	-	-
MIRU10	53	3	11	69	-	142	59	3	2	-	-	-	-	1
MIRU16	53	2	7	14	-	186	53	27	-	-	-	-	-	-
MIRU23	53	6	-	-	-	3	7	223	54	-	-	-	-	-
MIRU26	51	3	1	5	-	6	10	27	40	111	71	14	2	-
MIRU27	53	3	1	29	-	166	91	-	-	-	-	-	-	-
MIRU39	53	2	3	47	-	183	54	-	-	-	-	-	-	-
MIRU40	54	1	3	23	10	241	7	3	-	-	-	-	-	-
Mtub21	57	2	19	8	-	12	39	82	107	9	11	-	-	-
Mtub30	58	2	-	60	-	-	123	104	-	-	-	-	-	-
Mtub39	58	5	-	27	-	12	205	38	5	-	-	-	-	-

Table 3. First-Line Drug Resistant Frequency among 287 Clinical *M. tuberculosis* Strains

Drugs	No. (%) of Isolates				Area of Isolates			
	Beijing (*n*=230)	non-Beijing (*n*=57)	χ^2	*P*	Pizhou	Xinyi	Suining	Peixian
Monodrug resistance RFP	1 (0.43)	1 (1.75)	0.03	>0.05	2	0	0	0
INH	7 (3.04)	7 (12.28)	6.53	<0.05	4	3	3	4
SM	9 (3.91)	1 (1.75)	0.15	>0.05	4	1	4	1
Two-drug resistance								
RFP+INH	2 (0.87)	0 (0)		>0.05[1]	0	1	0	1
INH+SM	8 (3.48)	1 (1.75)	0.06	>0.05	5	1	2	1
Three-drug resistance								
RFP+INH+SM	5 (2.17)	0 (0)		>0.05[1]	1	1	3	0
INH+SM+EMB	1 (0.43)	1 (1.75)	0.03	>0.05	1	0	1	0
Four-drug resistance								
RFP+INH+SM+EMB	2 (0.87)	0 (0)		>0.05[1]	0	0	2	0
Total	35 (15.22)	11 (19.30)	0.57	>0.05	17	7	15	7

Note. RFP: rifampicin; INH: isoniazid; SM: streptomycin; EMB: ethambutol; [1]: Fisher exact probability.

In summary, the combination of Spoligotyping and 15 VNTR loci in our study was suitable for the genotype and it is helpful for controlling the dissemination of the strains in this area. However, we still need to explore better loci with less numbers or higher discrimination as the first line molecular genotyping in the future. Most of the drug resistant strains were of Beijing family. Consequently, the association as well as the special mechanism behind the genotypes and drug resistance should be further studied.

We wish to express our thanks to the staffs in the Xuzhou Municipal Center for Disease Control and Prevention for their efforts to collect specimens.

[#]Correspondence should be addressed to: 1) WAN Kang Lin, Tel: 86-10-58900776, E-mail: wankanglin@icdc.cn; 2) YANG Jin chuan, E-mail: xzyic@126.com

Biographical note of the first author: PANG Hui, female, born in 1981, Lecturer, PhD Candidate, majoring in studying epidemiology and drug resistance of *Mycobacterium*.

Received: June 27, 2014;

Accepted: October 11, 2014

REFERENCES

1. World Health Organization Global tuberculosis report 2013. Geneva, Switzerland: World Health Organization.
2. Liu JJ, Yao HY, Liu EY. Analysis of factors affecting the epidemiology of tuberculosis in China. Int J Tuberc Lung Dis, 2005; 9, 450-4.
3. Lu B, Dong HY, Zhao XQ, et al. A new multilocus sequence analysis scheme for Mycobacterium tuberculosis. Biomed Environ Sci, 2012; 25, 620-9.
4. Kamerbeek J, Schouls L, Kolk A, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol, 1997; 35, 907-14.
5. Cowan LS, Diem L, Monson T, et al. 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.
6. Wan KL, Liu JH, Hauck Y, et al. Investigation on Mycobacterium tuberculosis Diversity in China and the Origin of the Beijing Clade. PLoS One, 2011; 6, e29190.
7. Wendy Somerville, Louise Thibert, Kevin Schwartzman, et al. Extraction of Mycobacterium tuberculosis DNA: a Question of Containment. J Clin Microbiol, 2005; 43, 2996-7.
8. Small PM, Hopewell PC, Singh SP, et al. The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods. N Engl J Med, 1994; 330, 1703-9.
9. Dong H, Shi L, Zhao X, et al. Genetic Diversity of *Mycobacterium* tuberculosis Isolates from Tibetans in Tibet, China. PLoS One, 2012; 7, e33904.
10. Shamputa IC, Lee J, Allix-Béguec C, et al. Genetic diversity of *Mycobacterium* tuberculosis isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol, 2010; 48, 387-94.