ORIGINAL ARTICLE

Molecular typing of *Mycobacterium tuberculosis* isolates from Qinghai province of northwest china by spoligotyping and 15-locus MIRU-VNTR

Bin Li[1], ZhaofenWang[1]* , Yongcheng Ma[2], Haican Liu[3], Xiuli Shen[1], Binzhong Ma[2], Mingxia Jiang [2],Yuanyuan Zhang[1], Xiaodong Su[1], Wenqi Du[1], Haijing Wang[1], Xiuqin Zhao[3], Kanglin Wan[3]

Li B,Wang Z, Ma Y et al. Molecular Typing of Mycobacterium tuberculosis Isolates from Qinghai Province of Northwest China by Spoligotyping and 15-Locus MIRU-VNTR. J Microbiol Biotechnol Rep. 2018;2(1):1-4.

Tuberculosis (TB) is a major public health concern in Qinghai which located in the northeastern region of Qinghai-Tibet Plateau and characterized by low oxygen and low pressure. This study was conducted to explore the genotypes of Mycobacterium tuberculosis (MTB) in this high altitude region. A total of 251 MTB strains were collected and genotyped by spoligotyping and 15-loci mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing. Spoligotyping of 251 MTB isolates revealed 49 distinct spoligotype patterns and Beijing lineage was found to be the most dominant MTB strain including 74.0% (185/251) of total isolates. 217 isolates grouped into 15 clusters with the clustering rate of 86.5% (217/251). While 15-loci MIRU-VNTR distributed 251 isolates into 4 groups containing 238 different MIRU-VNTR profiles, Group II was the largest group representing 91.9% (230/251) of isolates. Clustering rate by MIRU-VNTR was 85.7% (215/251). Spoligotyping and MIRU-VNTR matched well in lineage classification. We concluded that 15-loci MIRU-VNTR has more abundant diversity than spoligotyping and would be useful to trace transmission routes and sources of infections.

Key Words: *Qinghai-Tibet Plateau; Mycobacterium tuberculosis (MTB); Spoligotyping*

INTRODUCTION

Tuberculosis (TB) is a common contagious disease of human beings, caused by bacillus *Mycobacterium tuberculosis* (MTB), which caused an estimated 10.4 million new TB cases and 1.8 million deaths worldwide in 2015. Most of the estimated number of cases occurred in developing countries, mainly in Asia (61%) and African Region (26%). China is one of 30 high TB burden countries by incident cases in 2015 just after India, Indonesia (1). In China, TB is a major public health problem with the highest absolute number of cases worldwide. Qinghai province lies on the Qinghai-Tibet Plateau with an average altitude of over 3000 meters, which results in low oxygen and low pressure in the region. This province is highly endemic for MTB, among 5.8 million people in this province, about 1.7 million people infected with TB and most of them were rural patients. Several papers have reported geographically structured populations in human pathogens (2-6). Unique geographical location and harsh natural environment and long-lived inhabitants of Tibetan in Qinghai may probably lead to adaptive genetic changes between hypoxia environment, host and pathogen of *Mycobacteria tuberculosis*. Based on this assumption, we conducted molecular studies among isolates of *Mycobacterium tuberculosis* circulating in Qinghai Plateau by Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) and spoligotyping.

MIRU-VNTR analysis (7-9) is a simple and quick genotyping method with a similar discriminatory power to that of RFLP (10). Thus the optimized 15 to 24-locus MIRU-VNTR typing system has been proposed for international standardization (11). Spoligotyping is a rapid and reliable method which based on DNA polymorphisms within the direct repeat (DR) locus of *M. tuberculosis* complex and is well suited for the identification of Beijing family *M. tuberculosis* strains (12). The sequencing and comparison of the genomes of *M. tuberculosis* H37Rv and *Mycobacterium* bovis has revealed large sequence polymorphisms (LSPs),which allowed the recognition of main phylogeographical lineages, resulting from the evolution of the *M. tuberculosis* complex members in association to their host populations (13-15) and RD105 was found to be specific to W-Beijing isolates (16).

Although molecular papers on *M. tuberculosis* have been carried out in China, no genetic diversity study was reported in Qinghai-Tibet plateau. In this study, spoligotyping and 15-locus MIRU-VNTR typing methods were applied for fingerprinting among 251 MTB isolates collected in Qinghai to better understand molecular characteristics and genetic diversities of MTB in this region.

MATERIALS AND METHODS

Clinical isolates of *M. tuberculosis*

A total of 251 clinical isolates of *M. tuberculosis* were from the Qinghai Center for Disease Control and Prevention. H37Rv from Chinese Center for Disease Control and Prevention (CDC) were used as reference control.

Culture, identification and genomic DNA extraction

All clinical isolates cultured in conventional Lowenstein-Jensen (L-J) media at 37°C for 6 to 8 weeks and were identified by means of standard microbiological and biochemical methods (17). Colonies of *M. tuberculosis* was collected from medium using the sterilized saline water and inactivated at 80 for 30 minutes. Precipitated bacteria by centrifugation for 3 minutes at 12000 rpm and then suspended with sterilized saline water and heated in water at 100°C for 1 hour. Supernatants containing the genomic DNA were collected by centrifugation for 3 minutes at 12000 rpm.

Genotyping methods

(1) Spoligotyping

Spoligotyping was performed by amplifying the whole Direct Repeat (DR) region basically as described by Kamerbeek et al. (18). The amplified PCR products were hybridized with nitrocellulose membrane having covalently linked 43 spacer oligonucleotides following the manufacturer's instructions. The results of spoligotyping were compared in binary format with the SpolDB4 database (http://www.pasteur-guadeloupe.fr/tb/spoldb4).

(2) VNTR typing

VNTR typing was performed by PCR amplification of 15 loci (MIRU10,

[1]*The Medical School of Qinghai University, Xining, P. R. China,* [2]*Institute for Communicable Disease Control and Prevention,Qinghai Provincial Center for Disease Control and Prevention, Xining, P.R. China,* [3]*State Key Laboratory for Infectious Diseases Prevention and Control, National Institute for Communicable Disease Control and Prevention and National Tuberculosis Reference Laboratory, Chinese Center for Disease Control and Prevention, Beijing, P.R. China*

Correspondence: Zhaofen Wang, The Medical School of Qinghai University, Xining, P.R. China. Telephone +86-0971-6104093, e-mail kristy538@163.com

Received: October 25, 2017, Accepted: January 10, 2018, Published: January 19, 2018

MIRU16, MIRU23, MIRU26, MIRU27, MIRU39, MIRU40, ETRA, ETRB, ETRC, ETRD, ETRE and Mtub21, Mtub30, Mtub39) as described by K. Wan et al. (19). The results were analyzed by Bionumerics 5.0 software.

RESULTS

Genotypes by spologityping

251 isolates produced a total 49 distinct spoligotype patterns. Of them, 7 spoligotypes were identified by SpolDB4.0. However, the other 42 types have not been described previously in the database. Of the 251 isolates, 156 (62.20%) belonged to Beijing family, and 9 (3.60%) were U genotype, 8 (3.20%) were MANU2 genotypes, 3 (1.20%) were T1 genotypes, 1 (0.40%) was H4 genotype, 2 (0.80%) were Beijing-like genotypes and other 27 (10.80%) belonged to Beijing-like genotypes. In addition, strains from other families, such as in the SpolDB4.0 database and referred to 45 (17.93%) 'new' genotypes (Figure 1).

Allelic diversity of the MLVA- VNTR

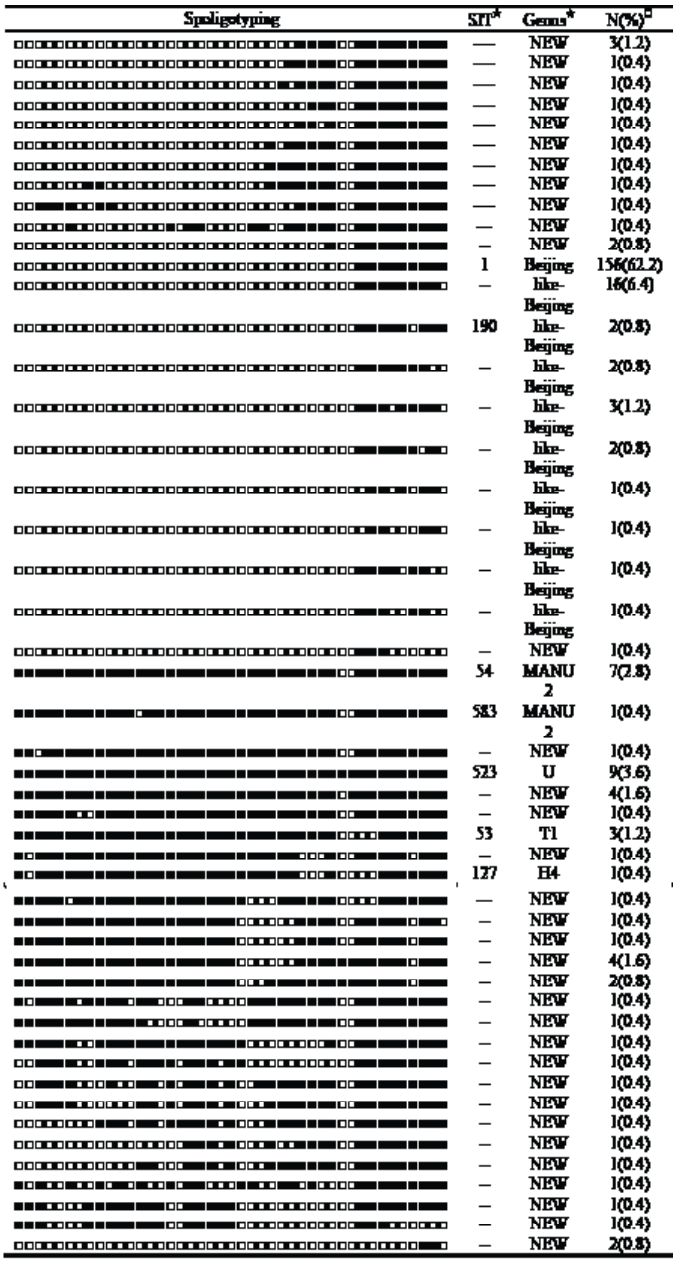
251 isolates were detected diversity by utilizing 15-loci MIRU-VNTR. They were distributed to 4 groups containing 238 strains genotypes (Figure 2). Hunter-Gaston discriminatory index (HGDI) of 15 VNTR loci displayed different powers ranging from the highest 0.769 for MIRU26 to the lowest 0.123 for ETRB. Six loci (ETRE, MIRU10, MIRU26, MIRU39, MIRU40, Mtub21) showed high discriminatory power (HGDI＞0.6). While four loci (ETRA, ETRD, MIRU27, Mtub39) demonstrated moderate discriminatory power (HGDI form 0.3 to 0.6), and five loci (ETRB, ETRC MIRU16, MIRU23, Mtub30) were poorly discriminatory (HGDI＜0.3), (Table 1).

Comparison of deligotyping and MLVA-VNTR

We compared the genotypes based on spoligotyping and MIRU-VNTR and found they showed excellent concordance (Table 2). Both methods displayed six families including Beijing, like-Beijing, U, MANU2, T1, H4 and the remaining 45 isolates were characterized with novel patterns not previously described grouped into New family. Beijing family was found to be the most abundant family in the studied population.

DISSCUSION

The global dissemination of Beijing genotype of *Mycobacterium tuberculosis* makes it a major issue of public health. *M. tuberculosis* of Beijing/W widely spread in many regions of the world, especially in Asia (20-22). Many countries, China, Mongolia, South Korea, Hong Kong, Malaysia and



Spoligotyping	SIT*	Genus*	N(%)□
	—	NEW	3(1.2)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	2(0.8)
	1	Beijing	156(62.2)
	—	like-Beijing	16(6.4)
	190	like-Beijing	2(0.8)
	—	like-Beijing	2(0.8)
	—	like-Beijing	3(1.2)
	—	like-Beijing	2(0.8)
	—	like-Beijing	1(0.4)
	—	like-Beijing	1(0.4)
	—	like-Beijing	1(0.4)
	—	like-Beijing	1(0.4)
	—	NEW	1(0.4)
	54	MANU2	7(2.8)
	583	MANU2	1(0.4)
	—	NEW	1(0.4)
	523	U	9(3.6)
	—	NEW	4(1.6)
	—	NEW	1(0.4)
	53	T1	3(1.2)
	—	NEW	1(0.4)
	127	H4	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	4(1.6)
	—	NEW	2(0.8)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	1(0.4)
	—	NEW	2(0.8)

Figure 1) *Spoligtypes of M. tuberculosis isolates (n=251)*

Note: ▪:representative cross site ☆:SIT from SpolDB4.0;★:spoligotype genus in the spolDB4.0; □:the number of the strains with the same SIT.

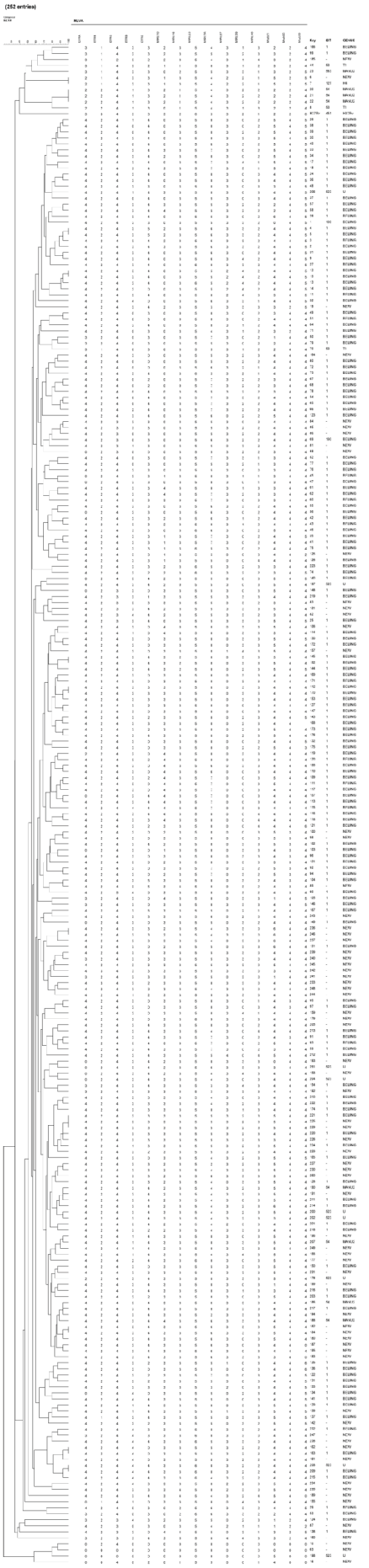


Figure 2) *MLVA-VNTR genotyping results for 251 MTB isolates*

Vietnam have high proportion of Beijing/W isolates (23-25). In this study, we assessed the prevalence of *M. tuberculosis* Beijing genotype by spoligotyping and MIRU-VNTR.

Previous studies reported that Beijing family strains in northern China was higher than that in the southern region and might relate to geographical location, climate, population composition and other factors (26). Spoligotyping of 251 isolates were grouped 185 (74.0%) into Beijing family and 66 into non-Beijing family, the former was significantly higher than the later. This suggests that the Beijing genotypes are dominant in Qinghai, which is similar to reported papers (19,27). 251 isolates revealed 49 spoligotyping profiles and distributed 217 isolates into 15 clusters with the clustering rate of 86.5% (217/251) (Figure 2). 34 isolates were not clustered. We observed a low diversity (*D*) among tested isolates which was calculated by dividing the number of different patterns by the number of isolates analyzed (28). The estimated degree of diversity was 19.5 (*D*=49/251 × 100) by spoligotyping. 61 isolates with novel spoligotyping patterns need to be further studied in the future.

VNTR loci were reported could be good markers for phylogenetic estimation (29). In this study, we distributed 251 isolates into 4 groups containing 238 different genetic patterns by 15-locus VNTR analysis (Figure 2). The estimated degree of diversity was 94.8 (*D*=238/251 × 100) which was much higher than that of spoligotyping, and the clustering rate was 85.7%(215/251) which was well matched with 86.5% by spoligotyping. This high genetic diversity of MTB strains by 15- locus VNTR demonstrates that Multiple-Locus Variable number tandem repeat Analysis (MLVA) would be useful for tracing transmission source.

Group II was found be the largest group in Qinghai province with 91.9 %(230/251) isolates, which was different from Ningbo of China, where the Group III was the main group with 65.6% of tested isolates (30). The founding suggests us more research need to do on pathogenicity, virulence and drug resistance among Group II MTB isolates which would be helpful in better control of MTB in this geographic region.

In conclusion, this is the first study to explore the molecular diversity of *M. tuberculosis* in Qinghai plateau by different typing methods. Our findings in current study may shed light on understanding molecular epidemiology and may allow the development of better strategies for TB control in this region.

TABLE 1

HGI index of VNTR-Loci for 251 *M. tuberculosis* Clinical trains

VNTR loci	All strains HGI	Beijing family HGI
ETRA	0.316	0.24
ETRB	0.122	0.07
ETRC	0.149	0.07
ETRD	0.436	0.39
ETRE	0.716	0.73
MIRU10	0.686	0.69
MIRU16	0.215	0.15
MIRU23	0.127	0.12
MIRU26	0.768	0.76
MIRU27	0.495	0.52
MIRU39	0.728	0.74
MIRU40	0.647	0.65
Mtub21	0.677	0.65
Mtub30	0.259	0.18
Mtub39	0.435	0.44

TABLE 2

Comparison of spoligotyping and MLVA-VNTR [n(%)]

Family	Spoligotyping	15 VNTR-locis
Beijing	156 (62.1)	156 (62.1)
like-Beijing	29 (11.6)	29 (11.6)
U	9 (3.6)	9 (3.6)
MANU2	8 (3.2)	8 (3.2)
T1	3 (1.2)	3 (1.2)
H4	1 (0.4)	1 (0.4)
New	45 (17.9)	45 (17.9)

FUNDING

This work was supported by grant from the National Natural Science Foundation of China (No.81160356 and No.81660556)

CONFLICT OF INTEREST

The authors of this paper declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank all the people who made their contributions in collecting samples and conducting phenotypic tests for identification.

REFERENCES

1. Organization WH World Health Organization Global Tuberculosis Report 2016. World Health Organization WHO Press Geneva Switzerland (2016).
2. Musser JM, Kroll JS, Granoff DM, et al. Global genetic structure and molecular epidemiology of encapsulated Haemophilus influenzae. Reviews of Infectious Diseases. 1990;12:75-111.
3. Agostini HT, Yanagihara R, Davis V, et al. Asian genotypes of JC virus in Native Americans and in a Pacific Island population: markers of viral evolution and human migration. Proceedings of the National Academy of Sciences of the USA. 1997;94:14542-46.
4. Falush D, Wirth T, Linz B, et al. Traces of human migrations in Helicobacter pylori populations. Science. 2003;299:1582-85.
5. Monot M, Honore N, Garnier T, et al. On the origin of leprosy. Science. 2005;308:1040-42.
6. Wirth T, Wang X, Linz B, et al. Distinguishing human ethnic groups by means of sequences from Helicobacter pylori: lessons from Ladakh. Proceedings of the National Academy of Sciences of the USA. 2004;101:4746-51.
7. Gagneux S, DeRiemer K, Van T, et al. Variable host–pathogen compatibility in *Mycobacterium tuberculosis*. Proceedings of the National Academy of Sciences of the USA. 2006;103:2869-73.
8. Frothingham R, Meeker-O'Connell WA. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology. 1998;144:1189-96.
9. Supply P, Mazars E, Lesjean S, et al. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol. 2000;36:762-71.
10. Supply P, Lesjean S, Savine E, et al. 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.
11. 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;44:4498-10.
12. Jia ZW, Jia XW, Liu YX, et al. Spatial analysis of tuberculosis cases in migrants and permanent residents Beijing 2000-2006. Emerg Infect Dis. 2008;14:1413-9.
13. Mostowy S, Cousins D, Brinkman J, et al. Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex. J Infect Dis. 2002;186:74–80.
14. Brosch R, Gordon SV, Marmiesse M, et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of the National Academy of Sciences of the USA. 2002;99:3684-89.
15. Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. Lancet Infect Dis. 2007;7:328-37.
16. Tsolaki AG, Gagneux S, Pym AS, et al. Genomic deletions classify the Beijing/W strains as a distinct genetic lineage of *Mycobacterium tuberculosis*. J Clin Microbiol. 2005;43:3185-91.
17. Kent PT , Kubica GP. Public health mycobacteriology : a guide for the level III laboratory. Centers for Disease Control and Prevention Atlanta. 1985.
18. Kamerbeek J, Schouls L, Kolk A, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J ClinMicrobiol. 1997;35:907-14.
19. Wan K, Liu J, Hauck Y, et al. Investigation on *Mycobacterium tuberculosis* diversity in china and the origin of the Beijing clade. PLoS One. 2011;6:e29190.
20. Bing Lu, Ping Zhao, Binbin Liu, et al. Genetic diversity of *Mycobacterium tuberculosis* isolates from Beijing, China assessed by spoligotyping, LSPs and VNTR profiles. BMC Infectious Diseases. 2012;12:372.
21. Bifani PJ, Mathema B, Kurepina NE, et al. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. 2002;10:45-52.
22. Glynn JR, Whiteley J, Bifani PJ, et al. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: A systematic review. Emerg Infect Dis. 2002;8:843-9.
23. Luo T, Comas I, Luo D, et al. Southern East Asian origin and coexpansion of *Mycobacterium tuberculosis* Beijing family with Han Chinese. Proceedings of the National Academy of Sciences of the USA. 2015;112:8136-41.
24. Chan MY, Borgdorff M, Yip CW, et al. Seventy percent of the *Mycobacterium tuberculosis* isolates in Hong Kong represent the Beijing genotype. Epidemiol Infect. 2001;127:169-171.
25. Park YK, Shin S, Ryu S, et al. Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of *Mycobacterium tuberculosis* in Korea. J Microbiol Methods. 2005;63:165-172.
26. Zhao Xiuqin, Dong Haiyan, Liu Zhiguang, et al. Preliminary Analysis of the Distribution of Beijing Genotype Strains of *Mycobacterium Tuberculosis* in Parts of China. J Practical Preventive Medicine. 2012;19:662-64.
27. Yu Q, Su Y, Lu B, et al. Genetic diversity of *Mycobacterium tuberculosis* isolates from Inner Mongolia, China. J PLoS ONE. 2013;85:e57660.
28. Zamani S, Haeili M, Nasiri MJ, et al. Genotyping of *Mycobacterium tuberculosis* Isolates from Hormozgan Province of Iran Based on 15-Locus MIRU-VNTR and Spoligotyping. International Journal of Bacteriology. 2016;7146470.
29. Wada T, Iwamoto T. Allelic diversity of variable number of tandem repeats provides phylogenetic clues regarding the *Mycobacterium tuberculosis* Beijing family. Infect Genet Evol. 2009;9:921-6.
30. Che Yang, Dong Hongjun, Yu Mei. Genotyping of *Mycobacterium tuberculosis* strains isolated from the patients in Ningbo with variable number tandem repeat method. Chinese Journal of Health Laboratory Technology. 2010;20:3299-301.