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Dominant modern sublineages and a new modern sublineage of *Mycobacterium tuberculosis* Beijing family clinical isolates in Heilongjiang Province, China

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ABSTRACT

Mycobacterium tuberculosis Beijing family includes a variety of sublineages. Knowledge of the distribution of a certain sublineage of the Beijing family may help to understand the mechanisms of its rapid spread and to establish an association between a certain genotype and the disease outcome. We have previously found that *M. tuberculosis* Beijing family clinical isolates represent approximately 90% of the clinical isolates from Heilongjiang Province, China. To clarify the distribution of *M. tuberculosis* Beijing family sublineages in Heilongjiang Province, China and to investigate the regularity rule for their evolution, we examined single nucleotide polymorphisms (SNPs) of 250 *M. tuberculosis* Beijing family clinical isolates using 10 SNP loci that have been identified as appropriate for defining Beijing sublineages. After determining the sequence type (ST) of each isolate, the sublineages of all *M. tuberculosis* Beijing family isolates were determined, and phylogenetic analysis was performed. We found that 9 out of the 10 SNP loci displayed polymorphisms, but locus 1548149 did not. In total, 92.8% of the isolates in Heilongjiang Province are modern sublineages. ST10 is the most prevalent sublineage (ST10 and ST22 accounted for 63.2% and 23.6% of all the Beijing family isolates, respectively). A new ST, accounting for 4% of the Beijing family isolates in this area, was found for the first time. Each new ST isolate showed a unique VNTR pattern, and none were clustered. The present findings suggest that controlling the spread of these modern sublineages is important in Heilongjiang Province and in China.

1. Introduction

The *Mycobacterium tuberculosis* (*M. tuberculosis*) Beijing family has spread widely in many countries and regions in the world (Bifani et al., 2002; Brudey et al., 2006; Hanekom et al., 2011) since it was first identified in 1995 (van Soolingen et al., 1995). Its wide spread has meant that great pressure has been brought to bear on the control of tuberculosis in China and world-wide. However, to date, the mechanisms for the rapid transmission of *M. tuberculosis* Beijing family are still unclear.

It has been claimed that *M. tuberculosis* Beijing family strains are resistant to Bacillus Calmette-Guerin (BCG) vaccination (Colditz et al., 1994; Parwati et al., 2010b), are highly virulent (Parwati et al., 2010a) and are associated with drug resistance (Almeida et al., 2005; Ghebremichael et al., 2010; Kremer et al., 2005; Tanveer et al., 2008). However, less association between this genotype and drug resistance has been reported in other geographic settings (Alonso et al., 2010; Anh et al., 2000; Jou et al., 2005; Toungoussova et al., 2003). An investigation regarding the epidemic of *M. tuberculosis* strains isolated in China revealed that Beijing family favors transmission but not drug resistance (Yang et al., 2012). We have also found that there is no difference in the drug resistance patterns between Beijing and non-Beijing genotype strains isolated in Heilongjiang Province, China, although the Beijing family represents approximately 90% of the clinical isolates in this area (Wang et al., 2011).

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The Beijing family contains a variety of subfamilies or sublineages (Filliol et al., 2006). The significance of genotyping always provides insights for phenotype, disease outcome and sources of infection. Knowledge of the distribution features of a certain sublineage of the Beijing family may improve our understanding of the mechanisms of its rapid spread and establish an association between a certain genotype and disease outcome.

Single nucleotide polymorphisms (SNPs) are used for genetically classifying *M. tuberculosis* and identifying sublineages. Because horizontal gene transfer or genetic recombination among different strains of *M. tuberculosis* complex (MTBC) is rare, the changes in genetic information are almost always obtained though hereditary from parental strains (Baker et al., 2004; Huard et al., 2006; Stucki et al., 2012). Therefore, SNPs have been used to classify *M. tuberculosis* Beijing family into different sequence types (STs) and is considered to be a robust target for defining the accurate position of a certain strain in a phylogenetic tree (Chen et al., 2012; Faksri et al., 2011; Filliol et al., 2006; Iwamoto et al., 2012; Mestre et al., 2011; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b).

To date, ancient and modern sublineages have been identified by SNPs. Though modern STs are dominant, the trends in the distribution of certain sublineages are geographically variable (Chen et al., 2012; Filliol et al., 2006; Iwamoto et al., 2012). For instance, a modern sublineage, ST10, prevails in Chongming Island, Taiwan, Thailand, and Peru (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Qiao et al., 2010), while an ancient type, ST19, is the dominant sublineage in Japan (Nakanishi et al., 2013; Wada et al., 2009b). However, the worldwide distribution and the epidemiological significance of the sublineages have not been well clarified yet. Extensive investigations regarding the possible reasons for the predominant prevalence of certain sublineages are needed to explore the significance of the variable distribution, the correlation between the evolution of *M. tuberculosis* Beijing family and its transmission patterns, and the impact on the spread of tuberculosis.

China is a country with a high burden of tuberculosis (TB). Heilongjiang Province, located in northeastern China, is one of the regions where the prevalence of both TB and drug-resistant TB is higher than the average level in China. We have found that *M. tuberculosis* Beijing family clinical isolates are the dominant TB strains in Heilongjiang Province (Wang et al., 2011). However, no correlation between the prevalence of Beijing family strains and general drug resistance has been found (Wang et al., 2011). It is urgent to understand possible mechanisms behind the spread of *M. tuberculosis*, especially drug-resistant strains.

In the present study, our aim was to discover the distribution features and proportions of *M. tuberculosis* Beijing family sublineages in Heilongjiang Province. We also aimed to define appropriate SNP loci for analyzing the sublineages of locally prevalent *M. tuberculosis* Beijing family clinical isolates. The findings will facilitate our understanding of the possible reasons for the predominant prevalence of certain sublineages and the highly epidemic of Beijing family in this area.

2. Materials and methods

2.1. M. tuberculosis clinical isolates

All the *M. tuberculosis* clinical strains were isolated from the patients from various regions of Heilongjiang province who were diagnosed with pulmonary TB at Harbin Chest Hospital. From June 2007 to November 2009, a total of 300 isolates were collected. Among them, 269 isolates were identified as *M. tuberculosis* Beijing family strains. Of these, 250 that had enough DNA for genotyping, including 60 from 2007, 107 from 2008, and 83 from 2009, were used in this study. *M. tuberculosis* H37Rv was used as the reference strain. All the patients were HIV-1 negative, and 68.4% (171/250) were male.

2.2. SNP typing

DNA extraction, molecular identification of *M. tuberculosis*, and the identification of Beijing family strains were carried out as described in our previous study (Wang et al., 2011).

The ten SNP loci shown in Table 1 were chosen because they have established polymorphisms among *M. tuberculosis* Beijing family strains and have been used for analyzing Beijing sublineages (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b). Polymerase chain reaction (PCR) amplification was carried out using the primers of Nakajima et al. (2013). Each PCR mixture was prepared in a volume of 50 μL containing 50 ng of genomic DNA, 2× Taq PCR Master Mix 25 μl (Nuo Weisen Biotech Co., Ltd., Beijing), and 0.2 μM of the corresponding primer. The PCR reactions were performed as follows: 75 °C for 5 min, 30 cycles of 75 °C for 10 s, 53 °C for 10 s, and 72 °C for 20 s, and final extension at 72 °C for 5 min. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. using the dideoxy chain termination method.

Nucleotide blast was carried out online (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and any differences between clinical isolates and the sequence of the reference $H_{37}Rv$ genome were identified. STs were determined based on the database and as previously described elsewhere (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b).

2.3. Identification of modern/ancient Beijing strains

It has been found that all modern Beijing strains of *M. tuberculosis* carry a mutation in codon 58 of the mutT2 gene (Hanekom et al., 2007; Iwamoto et al., 2008; Luo et al., 2012). Thus, a part of the mutT2 gene containing codon 58 was amplified. The primers included MutT2-Mut, (AGAGCTCGCCGAAGAACCGC, Forward), MutT2-Wt, (AGAGCTCGCCGAAGAACCGG, Forward), and MutT2-r (AAGCAGATGCACGCGATAGG, Reverse). Each strain was amplified using two pairs of primers: MutT2-Mut/MutT2-r (positive amplification only from mutants) and MutT2-Wt/MutT2-r (positive amplification only from wild-type). Each PCR mixture was prepared in a volume of 20 μL containing 150 ng of genomic DNA, 2× Taq PCR Master Mix 12.5 μl (Nuo Weisen Biotech Co., Ltd.,

Table 1
SNP loci and the primers used in identifying the mutations.

Locus[a]	Nucleotide sequence	Product size (bp)
797736	Forward: GACGGCCGAATCTGACACTG	266
	Reverse: CCATTCCGGGTGGTCACTG	
909166	Forward: CGTCGAGCTCCCACTTCTTG	288
	Reverse: TCGTCGAAGTGGACGAGGAC	
1477596	Forward: GTCGACAGCGCCAGAAAATG	232
	Reverse: GCTCCTATGCCACCCAGCAC	
1548149	Forward: GGCCAAGCCGTGTATTAGGG	306
	Reverse: AGTCGGCAGTGACGTTCTCG	
1692069	Forward: GATTGGCAACTGGCAACAGG	332
	Reverse: TGGCCGTTTCAGATAGCACAC	
1892017	Forward: GCTGCACATCATGGGTTGG	278
	Reverse: GTATCGAGGCCGACGAAAGG	
2376135	Forward: TCTTGCGACCCGATGTGAAC	373
	Reverse: GAGCGCAACATGGGTGAGTC	
2532616	Forward: CCCTTTTCTGCTCGGACACG	278
	Reverse: GATCGACCTTCGTGCACTGG	
2825581	Forward: CCTTGGAGCGCAACAAGATG	306
	Reverse: CTGGCCGGACGATTTTGAAG	
4137829	Forward: CGTCGCTGCAATTGTCTGG	229
	Reverse: GGACGCAGTCGCAACAGTTC	

[a] Position of SNP locus in $H_{37}Rv$ genome.