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# Prevalence and risk factors of mixed *Mycobacterium tuberculosis* complex infections in China

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**Summary** *Objectives:* Mixed infections have been considered as a potential obstacle for tuberculosis treatment and control. To date, few studies have been done to determine the rate of mixed infections of *Mycobacterium tuberculosis* in China.
*Methods:* In this study, we used the standard 24-loci MIRU-VNTR method to genotype the representative *M. tuberculosis* isolates from the national drug-resistant survey conducted in China. A total of 3248 *M. tuberculosis* complex (MTBC) strains had complete 24-loci MIRU-VNTR results and available for the analyses.
*Results:* Overall, MIRU-VNTR typing identified 115 (3.5%) isolates as being mixed MTBC infections in China. Statistical analysis revealed that mixed infections were significantly more likely to occur in men than women. Compared with the percentage of mixed infection from patients aged 45–56 years, the percentages of mixed infections were higher among patients aged 25–44 years [OR (95% CI): 1.844(1.129–3.014)] and old patients [older than 65 years OR (95% CI): 1.908(1.097–3.319)]. In addition, significantly higher frequencies of hemoptysis ($P = 0.022$) and chest pain ($P = 0.012$) were observed among mixed infections, using patients infected with a single strain as a reference.



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*Conclusions:* In conclusion, this study has provided the first comprehensive understanding of mixed MTBC infections in China, which will be essential to generate the effective TB control strategies.


## Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), is one of the leading causes of morbidity and mortality worldwide.[1,2] By the estimation from World Health Organization, 9.0 million new cases and 1.5 million deaths emerged globally in 2013.[1] Only behind India, China has the second highest burden of TB worldwide, accounting for 11% of global tuberculosis incidence.[1,3] In 2010, the fifth national TB survey of China revealed that the prevalence of pulmonary TB is 442 per 100 000 population.[2] Hence, the serious epidemic of TB has been a major threat from both a clinical and a public health perspective in China.[4]

Traditionally, infection by *M. tuberculosis* was believed to be caused by a single strain.[5–7] However, benefit from the application of molecular genotyping methods to differentiate *M. tuberculosis* strains, infection with multiple *M. tuberculosis* strains in a single host, has been increasingly demonstrated by several molecular-based studies.[8–10] In comparison with other DNA fingerprinting methods, Mycobacteria interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) is the most widely used method for the detection of mixed infections.[5] Unfortunately, the genotyping methods are widely used in the molecular epidemiological study rather than clinical practice. Due to the limited sensitivity of the current diagnosis approaches, mixed infection is difficult to be detected.[5] If the patients harbor both drug-susceptible and resistant *M. tuberculosis* strains, the minor drug-resistant population may be not detected by the existing drug susceptibility testing (DST) methods. The presence of underlying resistance therefore erodes treatment success and increases the risk of acquired resistance with standardized combination therapy.[5,11–13] The mixed *M. tuberculosis* infection, therefore, may play a crucial role on the effective treatment of individual and the control of tuberculosis in the community. In addition, numerous literature have documented that the frequency of mixed infections in human population differs in different settings, ranging from 1.2% to 57.1%.[5] The significantly diverse rate of mixed infection among various regions highlights the urgent need to realize these important data of different geographic settings, which will provide new insights to generate the optimized local strategy for tuberculosis control.

To date, few studies have been done to determine the rate of mixed infections of *M. tuberculosis* in China.[14] Thus, we used the standard 24-loci MIRU-VNTR method to genotype the representative *M. tuberculosis* isolates from the national drug-resistant survey conducted in China. Our aim was to explore the prevalence of mixed *M. tuberculosis* complex infections in the current setting. In addition, we sought to extend our findings on the relationship between demographic or clinical characteristics and mixed infections.

## Materials and methods

### Ethic statement

The study was approved by the Tuberculosis Research Ethics Review Committee of the China CDC. Written informed consent was obtained from each participant.

### Bacterial strains

A total of 3929 *M. tuberculosis* strains isolated from the national survey in China were enrolled in this study.[15] Two sputum samples for culturing were obtained from each eligible patient before the initiation of treatment, and Lowenstein-Jensen (L-J) solid medium was used for phenotypic assessment of growth. In addition, one positive culture with higher positive grade for each patient was selected for bacterial preservation. All the freshly grown colonies were scraped from the surface of the solid medium. The bacterial cells were stored in the National Tuberculosis Reference Laboratory. Prior to preparation of thermolysates, the strains were recovered on L-J medium for 4 weeks at 37 °C.

### Preparation of thermolysates

The thermolysates were prepared from freshly cultured bacteria using method as previously reported.[16] The bacteria cells were transferred to a microcentrifuge tube containing 500 μl Tris—EDTA (TE) buffer, followed by centrifugation at 13 000 rpm for 2 min. After the supernatant was discarded, the pellet was resuspended in 500 μl TE buffer and heated in a 95 °C water bath for 30 min. After centrifugation to remove cellular debris, the DNA in the supernatant was used for PCR amplification reactions. The absorbance of the crude DNA on 260 nm was detected by NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), which served as an indirect indicator of DNA yield.

### Genotyping

The standard 24-loci MIRU-VNTR typing method was carried out to determine the composition of strains isolated from patients as previously reported.[17] Briefly, analyses were performed by using multiplex PCR, Rox-labeled MapMarker 1000 size standard (BioVentures, Murfreesboro, TN) and ABI 3730XL DNA sequencer. The customized GeneScan (Applied Biosystems) was used to analyze the length of the PCR fragments and assignment of the various VNTR alleles.

The 20 μl PCR mixture was prepared as follows: 2 μl of PCR buffer, 1.5—3.0 mM of $MgCl_2$, 4 μl of Q-solution, 2 ng of genomic DNA, 0.2 μl of HotStartTaq DNA polymerase



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