# Mixed infection with Beijing and non-Beijing strains in pulmonary tuberculosis in Taiwan: prevalence, risk factors, and dominant strain

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## Abstract

Patients with pulmonary tuberculosis (TB) can be simultaneously infected with different strains of *Mycobacterium tuberculosis* (mixed infection). We investigated the prevalence and risk factors of mixed infection by Beijing and non-Beijing strains in pulmonary TB patients in Taiwan. We developed a quantitative PCR method to simultaneously detect the presence of Beijing and non-Beijing strains. A total of 868 pretreatment samples (from 868 patients), including 563 sputum samples smear-positive for acid-fast bacilli and 305 liquid medium samples culture-positive for mycobacteria, were tested. Medical records of patients with culture-confirmed pulmonary TB were reviewed. The detection limit of our quantitative PCR method was five copies of target sequences. With mycobacterial culture result as the reference standard, the sensitivity and specificity of our quantitative PCR method were 95% and 98%, respectively. *M. tuberculosis* strains were isolated in 466 samples, of which 231 (49.6%) were infected with a Beijing strain. Another 14 patients (3.0%) had mixed infection, with the Beijing strain being the dominant strain in 13 (93%). Age <25 years with pulmonary cavities was associated with mixed infection. In patients infected with non-Beijing strains, the bacterial load of non-Beijing strains was lower among those with mixed infection than among those without. Our quantitative PCR method was accurate in detecting Beijing and non-Beijing strains in smear-positive sputum and culture-positive liquid medium samples. Mixed infection was present in pulmonary TB patients (3.0%), especially in those aged <25 years with pulmonary cavities. Beijing strains seem to be more dominant than non-Beijing strains in patients with mixed infection.

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## Introduction

It has been commonly thought that tuberculosis (TB) is caused by a single strain of *Mycobacterium tuberculosis* [1,2]. However, studies have found that patients may be simultaneously infected with different strains (mixed infection) [3,4]. By amplifying and detecting DNA sequences of Beijing and non-Beijing strains, a study in Cape Town showed that 19% of all patients with TB were simultaneously infected with one Beijing and one non-Beijing strain, and that this occurred more frequently in retreatment cases (23%) [5]. Several other genotypic approaches based on detecting genomic regions specifically deleted in the Beijing strains have also been utilized to identify Beijing strain and mixed infection in clinical samples [6–8]. The presence of mixed infection can lead to conflicting results of drug susceptibility testing if both drug-susceptible and drug-resistant *M. tuberculosis* strains are present [9–11]. Drug-resistant strains may not be detected initially if susceptible strains are predominant, but may outnumber susceptible strains at a later point in time during anti-TB treatment. Furthermore, it was observed that a drug-susceptible strain re-emerged in patients with multidrug-resistant (MDR) TB treated with second-line anti-TB drugs [12]. Therefore, undetected drug-resistant strains in

the presence of drug-susceptible strains may result in unfavourable treatment outcomes.

The prevalence and risk factors of mixed infection in tuberculous populations have rarely been investigated, mainly because of technical difficulties. In Taipei, Taiwan, Beijing strains account for 50% of the clinical isolates of *M. tuberculosis* [13]. Therefore, the magnitude of mixed infection could be investigated by simultaneous detection of one Beijing and one non-Beijing strain. As the incidence of TB in Taipei is not as high as that in the study sites of Cape Town, we thought that mixed infection in TB is less frequent in Taipei, but hypothesized that it was not undetectable. Therefore, we conducted a study to investigate the prevalence and risk factors of simultaneous infection with Beijing and non-Beijing strains in patients with pulmonary TB by using a real-time quantitative PCR (Q-PCR) method. We report the results of this study.

## Materials and Methods

### Materials and protocols

This prospective study was conducted in a 700-bed medical centre in northern Taiwan. Pretreatment sputum samples from patients suspected of having TB that were either smear-positive for acid-fast bacilli or culture-positive for mycobacteria were collected from July 2007 to December 2008 in the mycobacteriology laboratory, which is a regional reference laboratory whose quality is periodically assessed by the national reference laboratory of the Taiwan Centers for Disease Control. Only one specimen was collected from each patient.

Mycobacteriological studies were performed as previously described (see Data S1) [14,15]. For sputum samples that were smear-positive for acid-fast bacilli, or culture-positive in liquid medium, we immediately extracted genomic DNA [16] and performed a PCR test with the Cobas Amplicor MTB system (Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer's instructions. The results of the PCR test were analysed with the Cobas Amplicor Analyzer (Roche Instrument Center AG, Rotkreuz, Switzerland).

Medical records of patients with pulmonary TB confirmed by both mycobacterial culture and Q-PCR test were reviewed to obtain the demographic data, history of previous TB, and chest radiographic findings.

### Procedures of real-time Q-PCR

For each extracted genomic DNA, a Q-PCR test was also performed to detect mixed infection, defined as the simultaneous presence of a Beijing and a non-Beijing strain. If the Q-PCR test revealed mixed infection, we then performed a PCR with the same primer sets, and sequenced the amplicons on an ABI Prism 3730 DNA sequencer with a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) to confirm their presence.

Two primer sets and probes were designed to detect the Beijing and non-Beijing strains of *M. tuberculosis* in the samples (see Fig. S1). The primer sequences for the Beijing strains were complementary to the 3′-end of the IS*6110* element and Rv2820. A positive Q-PCR signal indicated the presence of an IS*6110* insertion in Rv2820, which is unique to the Beijing evolutionary lineage [17]. The primer sequences for non-Beijing strains were complementary to Rv2819. The standard curve for calculating the number of copies of each DNA sequence in the sample was generated with different concentrations of cloned plasmid containing the target sequence (see Data S1).

To assess the reproducibility of Q-PCR, two independent experiments were performed for each sample.

### Minimizing laboratory cross-contamination

Several steps were applied to minimize the possibility of laboratory cross-contamination. First, fewer than five samples were processed at the same time. Second, acid-fast smears, mycobacterial cultures, DNA extraction and Cobas Amplicor MTB assays were performed in a biosafety level 2-plus laboratory, and the Q-PCR was performed in a biosafety level 2 laboratory. For each Q-PCR reaction, DNA from two different Beijing and two different non-Beijing strains, confirmed by spoligotyping [18], were used as positive controls, and de-ionized water as negative control.

### Statistical analysis

The reproducibility of the Q-PCR was evaluated by applying Pearson's correlation and *t*-test. Correlation between the results of Cobas Amplicor and Q-PCR was evaluated by calculating the kappa coefficient. Because of the potentially non-linear effect of age, the spline smoothing model was applied to evaluate its impact on mixed infection. Multivariate logistic regression analysis was used to identify risk factors of mixed infection, and linear regression analysis to identify factors influencing mycobacterial load (see Data S1). A two-sided p-value $<0.05$ was considered to indicate significance. All analyses were performed with SAS software (Version 9.1.3; SAS Institute, Cary, NC, USA).

## Results

A total of 868 samples, including 563 smear-positive sputum samples and 305 culture-positive liquid medium samples, col-