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The diversity of *Mycobacterium tuberculosis* strains in China: tracing the origins of the worldwide dispersion of the multidrug-resistant Beijing genotype

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Project web-site:

<http://bacterial-genotyping.igmors.u-psud.fr/TBChina/>

Summary:

In China a large fraction of the population is infected by *Mycobacterium tuberculosis*, the bacteria responsible for tuberculosis (TB), with 500.000 new cases every year. An important proportion of strains is resistant to multiple drugs used to treat this disease. The attenuated *Mycobacterium bovis* Bacillus Calmette Guerin (BCG) is the only available vaccine against TB. However this vaccine does not provide consistent protection against TB. Knowledge of the diversity of the *M. tuberculosis* strains is of major importance to identify *M. tuberculosis* antigens and epitopes for new protective vaccines and specific diagnostic reagents.

Previous work as well as our unpublished study on Chinese *M. tuberculosis* strains showed that the Beijing genotype is prevalent in China. Strains of a similar genotype are found worldwide (particularly in Eastern Europe and in the countries of the former Soviet Union) and are often associated with multidrug-resistance. The very rapid and efficient dispersion of the Beijing strains suggest that they may have developed selective advantages.

We are organizing a survey of *M. tuberculosis* genetic diversity in China which necessitates the analysis of a large collection of strains (6000) from the 31 Provinces. The data is compared to the characteristics of strains collected in other countries. Our goal is to characterize the dominant bacterial populations in China and to find whether the dispersion of the Beijing type is of clonal origin.

During the period of this SSA we prepared the project in collaboration with Chinese scientists and performed a survey in 12 provinces. For this the technical knowledge was transferred and the progress of the experiments was regularly assessed through exchanges of personals and meetings. The data has been collected in a shared-database and we are now producing population analyses.

During the first year we defined the characteristics of the patients to be included in the protocol and we chose the appropriate methods for collecting and storing strains, for genotyping and for multidrug-resistance (MDR) assessment. As a pilot assay we first concentrated our work on the Beijing Province, then we extended the study to more provinces reaching a total of 1200 strains collected and analysed. Data on the BCG status (when available), age and gender of the patients as well as other relevant clinical information were collected.

This work has been the subject of Wan Kanglin's PhD thesis under the co-supervision of Dr G. Vergnaud and Pr. Xu Jianguo (Université Paris-Sud, October 8th 2007). A manuscript is in preparation for publication.

Problem:

It is estimated that one third of the world's population is currently latently infected by *Mycobacterium tuberculosis*, the agent of tuberculosis (TB), mostly in poor countries. The extensive use of the BCG vaccine has not lead to eradication of this disease; on the contrary it may have allowed some strains to emerge, in particular in countries where the use of drugs was not adequate. In China a particular strain family called the Beijing family has been found to predominate (van Soolingen et al., 1995). Strains of this type could be traced back to the middle 1950s owing to the availability of biological samples, and are presumably much older. They have spread to other countries in Asia as shown by different studies, and Eastern Europe mostly in Russia and countries of the former Soviet Union. The so-called W strain described in New York, USA, belongs to the Beijing family.

Strains of the Beijing family have been found to develop a multidrug resistance with high frequency in some parts of the world (Kruuner et al., 2001). This may be due to their genetic background which would make these strains more adaptable to the irregular use of anti-TB treatments, or to the fast spreading of a few well-adapted clones owing to migrations of their human host. The majority of multidrug-resistant strains collected from patients in refugees camps by Médecins Sans Frontières belong to the Beijing family (our unpublished data).

In addition, studies by (Lopez et al., 2003) showed that bacteria with the Beijing genotype elicited a non-protective immune response in mice and were the most virulent.

Aim:

The "Tuberculosis China" SSA was intended to help in identifying emerging *M. tuberculosis* strains in order to determine their antigenic characteristics and eventually help develop a new protective vaccine. Very often genotyping of TB strains has been performed by scientists from developed countries (Europe, USA) using samples collected in poor countries. The objective of the SSA was to set the bases of a large epidemiological survey to be performed in China where TB is a major health problem, in a joint effort with European teams. Several studies on the worldwide dissemination of Beijing strains have been already conducted in Europe and in the USA (van Soolingen et al., 1995; Glynn et al., 2002; Bifani et al., 2002). TB strains from China

were analyzed; however this is the first time that a survey encompassing the totality of the Chinese provinces is undertaken.

Another important point was to understand how *M. tuberculosis* strains vary and adapt to new treatments or vaccines. It is not known why the Beijing family has been able to spread worldwide with such efficiency. Investigation on drug-resistance pattern is necessary as well as information on patients in order to perform epidemiological studies. This implied organizing the collect of samples all over China and deciding with the European partners of the protocols for testing drug-resistance and for genotyping. One task was the definition of a high quality genotyping procedure that can be used to type thousands of strains at a low cost. In order to transfer the necessary technologies for performing the analyses in China, we favoured robust PCR-based techniques that do not require high quality DNA and that can be performed in moderately equipped laboratories.

The Chinese Reference Laboratory for Tuberculosis in Beijing can collect strains and patient information in the different provinces of China. Together with the European partners, the Chinese scientists will then perform the different analyses to characterize the strains and will analyse the data. This is an ambitious project which necessitates serious organization and a long-term follow-up.

Achieved and expected results:

The genetic diversity of MTB strains

Although population movements are increasingly important within China with emigration from the rural regions to large cities, there has been little recent immigration from other countries which facilitates the search for ancestral MTB lineages. The genotyping of about 1200 strains from 12 provinces has allowed a description of prominent bacterial families and their distribution over the country. Interestingly the South of China is the place where the smaller percentage of Beijing-family strains is found (less than 60%) whereas in the North and the East up to 95% of strains belong to this family. Most of the other strains belong to two families of recent evolution and which are found almost exclusively in China. We have also uncovered a new family which may represent the ancestor of the Beijing family.

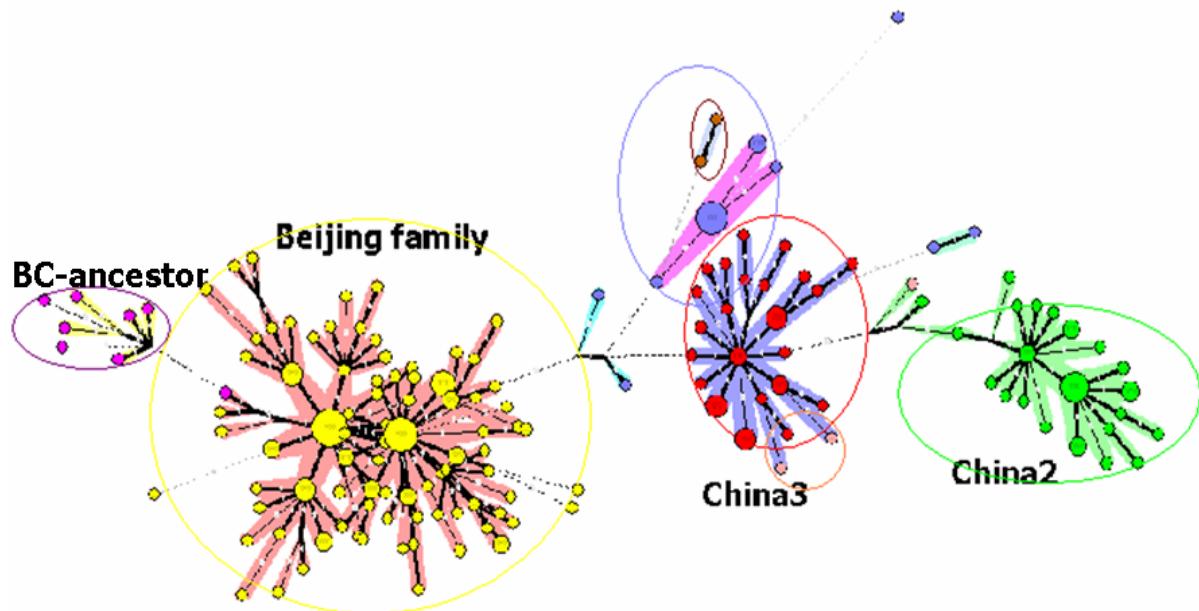


Figure 1: Analysis of *Mycobacterium tuberculosis* genetic diversity in China: Genotyping of the 202 *M. tuberculosis* strains shown here was performed using 15 VNTRs. The genetic profiles were compared using the Minimum spanning tree program, and the strains (represented by circles) were clustered according to their degree of similarity. The figure shows that a majority of strains represented by coloured circles are aggregated. The larger cluster in yellow corresponds to the Beijing family. Two large groups of isolates surrounded with green and red constitute two new families that we call China 2 and China 3 and which are found mostly in China. A new family in pink may represent the ancestor of the Beijing lineage.

The role of BCG vaccination in emergence of new TB strains

It is still not clear what was the role of BCG vaccination if any in allowing the emergence and spread of the Beijing family. Contradictory observations have been made (Le et al., 2000). The preliminary results of the present SSA suggest that BCG vaccination is not the cause for the emergence of the Beijing-family in China. However much remains to be done to get a statistical measure, principally because the BCG status of the patient is not always known. The difficulty of such a study is high particularly because the Chinese population is very large and multiple factors may influence the emergence of pathogenic bacteria.

Multidrug-resistance (MDR)

What is particularly preoccupying is the high percentage of multidrug-resistant strains in China. Our results show that in some provinces, one hospitalized patient out of three with tuberculosis possesses a MDR strain. This may be the consequence of inadequate use of antibiotics. To

confirm these results new tests should be performed using standardized protocols. We did not observe in the present study a relationship between multidrug resistance and the Beijing-family genotype.

Potential applications

The main objective in dealing with TB is to be able to develop new vaccines or treatments. This necessitates knowledge of the MTB strains which exist in poor countries and those that are disseminated, in particular through emigration. We have identified two new families of strains which account for the majority of non-Beijing family isolates in China. In addition other lineages exist in some provinces and may represent emerging clones. The genome of a representative strain of each new lineage will be sequenced in search for potential therapeutic targets.

Studies are being performed in Beijing to identify *M. tuberculosis* antigens and epitopes for new protective vaccines and specific diagnostic reagents. Immunogenicity and protective capacity may be increased by adding to the BCG strains antigens that induce a T-cell response (Pym et al., 2003).

Key words:

China, Tuberculosis, epidemiology, MLVA, genotyping, BCG, Beijing-family.

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Annexe

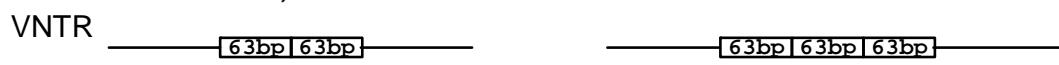
MLVA genotyping scheme using 15VNTRs (MLVA 15): ETR-A, ETR-B, ETR-C, ETR-D (MIRU04), ETR-E (MIRU31), MIRU10, MIRU16, MIRU23, MIRU26, MIRU27, MIRU39, MIRU40, Mtub21, Mtub30, Mtub39.

Setting an MLVA assay

Lanes 1, 9, 18, 27, size marker

Lanes 2, 10, 19, reference strain

All other lanes, test strains



Electrophoresis on agarose gel

