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Factors Underlying the Success of the *Mycobacterium tuberculosis* Beijing genotype in Indonesia

Ida Parwati
Factors Underlying the Success of the *Mycobacterium tuberculosis* Beijing genotype in Indonesia

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Factors Underlying the Success of the 
*Mycobacterium tuberculosis* Beijing genotype 
in Indonesia

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Factors Underlying the Success of the Mycobacterium tuberculosis Beijing genotype in Indonesia

An academic essay in Medical Science

Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. Dr. S.C.J.J Kortmann,
according to the decision of the council of deans
to be defended in public on Monday November, 9 2009
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Chapter 1

Introduction
**Introduction**

*Mycobacterium tuberculosis* is a highly successful pathogen that has plagued mankind and animals for thousands of years. Each year, there are an estimated nine million new cases of tuberculosis (TB) and about 1.8 million deaths due to TB. Although diagnostic tests, chemotherapy and vaccination are available, the disease is far from being eradicated. Recent data suggest that the propensity to gain drug resistance as well as the pathogen’s transmissibility profile may be influenced by the genetic and evolutionary background of *M. tuberculosis* strains. Therefore, understanding the relationships and dynamics of the *M. tuberculosis* complex lineages will undoubtedly help to control TB.

**The molecular epidemiology of tuberculosis**

The development of molecular techniques has allowed investigators to determine the genetic relatedness of *Mycobacterium tuberculosis* complex isolates. Previously it was believed that *M. tuberculosis* complex was genetically highly conserved, but on basis of an unprecedented DNA polymorphism, thousands of different *M. tuberculosis* strains have been recognised. A standardized methodology exploiting this polymorphism to discriminate *M. tuberculosis* strains was introduced in 1993 (1). This method, called restriction fragment length polymorphisms (RFLP) is based on a variable number of IS6110 insertion elements in the genome of different *M. tuberculosis* complex strains and the high degree of variation in the genomic sites of integration. The use of RFLP analysis has revolutionized outbreak investigations and conventional epidemiology (2). In particular, IS6110 RFLP typing has been used to generate fingerprints in various epidemiological studies of institutional and community outbreaks (3). However, this technique is laborious and time-consuming, and it has insufficient capacity to discriminate *M. tuberculosis* strains with low copy numbers of IS6110.

In 1991, an extensive polymorphism was observed between *M. tuberculosis* isolates in the direct repeat (DR) chromosomal region. This region comprises multiple identical 36-bp DR sequences interspersed by unique spacer regions ranging from 35 to 41 bp in length (4). Spacer oligonucleotide typing (also called: spoligotyping) is a simple technique to determine the presence or absence of 43 spacer regions in *M. tuberculosis* isolates, targeting the spacer sequences that separate the Direct Repeats (DRs) in the spacer interspersed direct repeats (SPIDRs). This technique was introduced in 1995 as an alternative fingerprinting technique (5). Spoligotyping has several advantages over IS6110-based genotyping. First, because it is a PCR based fingerprinting technique, the DNA can be of lower purity, which makes spoligotyping much easier to perform than other genotyping techniques. Second, because small amounts of DNA are required, it can be performed on bacteria in clinical samples or on strains of *M. tuberculosis* shortly after their inoculation into liquid culture or bacteria in Ziehl-
Neelsen slides (6). Third, the results of spoligotyping, which are expressed as positive or negative for each spacer, constitute a digital format, so that patterns can easily be compared and analyzed. Fourth, although spoligotyping gives a more limited level of strain discrimination than some other techniques, it is highly informative regarding the phylogeny of the \textit{M. tuberculosis} isolates. Therefore it has been used all over the world besides RFLP, culminating in a global database (7). Spoligo patterns have been used for phylogenetic studies, analyzing evolutionary relationships, and indirectly to unravel mechanisms of virulence of \textit{M. tuberculosis} complex strains.

Nowadays, another direct repeat driven fingerprinting technique has been introduced, namely Variable Number of Tandem Repeat (VNTR) typing. VNTR seems to be the most suitable typing technique for the coming period because it is PCR-based and offers sufficient discrimination for strain typing as well as phylogenetical analysis of \textit{M. tuberculosis} complex strains (8-10).

\textit{Mycobacterium tuberculosis} Beijing genotype

One clade of \textit{Mycobacterium tuberculosis}, the ‘Beijing genotype’ family, first described in 1995 (2), is one of the most successful clades in the current worldwide tuberculosis (TB) epidemic. The Beijing genotype was recognized on basis of the highly conserved spoligo patterns and characteristic IS6110 RFLP patterns of \textit{M. tuberculosis} isolates from the Beijing region and Mongolia. Since then, the Beijing genotype has been found in many different countries, especially in Asia, the former Soviet republics, and (South) Africa, but also Northern American cities. Typical RFLP and spoligo patterns of \textit{M. tuberculosis} Beijing strains are shown in Figure 1.
Beijing genotype strains represent group 1 of the original grouping of Sreevatsan based on a limited number of single nucleotide polymorphisms (12), but are considered a grouping of the “modern” lineage of \textit{M. tuberculosis} according to the grouping of the \textit{M. tuberculosis} complex based on large sequence polymorphisms (13). The Beijing clade consists of at least two major groupings; the “typical and “atypical” Beijing strains, which differ in their distribution in different countries and age groups, and which may have different properties. Typical (“modern”) Beijing strains, including \textit{W} strains, exhibit highly similar, multicopy IS\textsubscript{6110} RFLP patterns Atypical (“ancestral”) Beijing strains more closely resemble the common
ancestor of the Beijing clade (14-17). Beijing strains can also be subdivided in various evolutionary lineages through large sequence polymorphisms (LSPs), which help to divide the Beijing family into four monophyletic subgroups, on basis of so-called ‘Regions of Difference’ (RD), namely RD105, RD181, RD150, and RD142 (18). The Beijing genotype of *M. tuberculosis* has recently also been recognized as the major part of the ‘East-Asian lineage’ (19, 20).

The fourth International Spoligotyping Database (SpolBD4), which classified 39,295 strains of *M. tuberculosis* from 141 countries into 62 clades/lineages, has indicated that Beijing and Beijing-like strains represent at least 50% of strains in East Asia, and 13% of the isolates globally (7). However, because spoligotyping has mostly been used in Western countries, the data from many high prevalence settings are missing. It is possible that the true contribution of Beijing strains to the worldwide TB epidemic may even be higher.

A recent study determined the time of divergence, population diversity and spread of *M. tuberculosis* complex by MIRU-typing of a collection of 355 strains representing all well-defined primary branches of *M. tuberculosis* complex. Compared to other clades, the Beijing genotype displayed the largest population increase taken place in the last 180 years (21). Investigation of archived specimens in South Africa has confirmed the recent expansion of the Beijing genotype in the last decades. Spoligotyping was applied to paraffin-embedded clinical material from consecutive time periods. In this area, Beijing strains were absent in histological samples from the period 1930–1965, rare in samples from 1966–1995, but increasingly common in samples from the period 1996–2005, showing a 20% increase since 2000 (22).

Many studies have been conducted to investigate why the Beijing genotype has spread globally in a relatively limited time period. Different animal models have shown that Beijing strains are more virulent, causing more histopathology, higher outgrowth and increased mortality (23-25). Studies in human patients have found higher rates of drug resistance among Beijing strains in some, but not all geographic areas (26).[the systematic review..] Studies examining the virulence of *Mycobacterium tuberculosis* Beijing genotype have also met with conflicting results. Some studies found more radiological abnormalities (reflecting more extensive lung damage), while others did not. Some studies found a relationship with BCG-vaccination, suggesting that the Beijing genotype is an ‘escape’-variant, but others did not. A limited number of studies have linked the Beijing genotype with treatment failure, but most are cross-sectional and should therefore be interpreted with caution. The geographic variation of *M. tuberculosis* might also be related to the human population structure, but so far only one study has directly examined this hypothesis (27). Because the population structure of *M.
tuberculosis is still unclear in many areas, our understanding of mechanism underlying the success of this lineage of *M. tuberculosis* is far from complete.

**Tuberculosis in Indonesia**

After China and India, Indonesia has the third highest case load of tuberculosis, with more than half a million new cases per year, and an estimated 150,000 attributable deaths (28). However, very little research related to TB in Indonesia has focused on molecular and other aspects of its cause: *Mycobacterium tuberculosis*. For instance, there are hardly any data about the population structure of *M. tuberculosis* in this country; except for one small study including 92 isolates from a single clinic in Jakarta (11), no data have been published on *M. tuberculosis* genotypes in Indonesia. In addition, as culture is not routinely performed, and additional characterization of isolates, including drug susceptibility testing is rarely done, drug resistance data are lacking. Finally, no studies have examined the clinical phenotype of different *M. tuberculosis* genotype families.

**Thesis outline**

This thesis focuses on genetic analysis, risk factors and host interactions of *Mycobacterium tuberculosis* strains, especially those belonging to the Beijing genotype. The patients described in this thesis were recruited in Indonesia, partly in the context of a case-control study exploring immunogenetic determinants of susceptibility to tuberculosis (29). Most of the bioanalysis was conducted in Indonesia.

This thesis consists of three parts; the first part deals with the development and application of spoligotyping for the molecular epidemiology of *M. tuberculosis* in Indonesia (chapters 2 - 4). The second part focuses on the clinical phenotype of the Beijing genotype (chapters 5 and 6), and the third on possible explanations for its success (chapters 7 and 8).

In chapter 2 I examined the reproducibility of spoligotyping, implementing this as the first molecular technique in a clinical laboratory in Bandung, Indonesia. We encountered problems of reproducibility and examined how spoligotyping can be optimized for different purposes.

In chapter 3 I examined the population structure of *M. tuberculosis* in patient cohorts on two different Indonesian islands, to answer the question what proportion of patients was infected with Beijing genotype strains.

Drug resistance possibly contributes to the emergence of Beijing strains in different areas around the world. In Chapter 4 I examined if Beijing strains display
more-or-another distribution of mutations in drug resistance genes which account for resistance to three different tuberculosis drugs.

Chapter 5 and 6 describe the clinical phenotype of patients infected with *M. tuberculosis* Beijing genotype strains. In chapter 5 I examined if Beijing strains are associated with TB treatment failure.

In chapter 6 I examined the long term effects of pulmonary TB by comparing ex-TB patients with matched controls. Specifically, the question was addressed whether patients previously infected with Beijing genotype strains experience more recurrent TB or more lung damage than those previously infected with other genotype strains.

Chapter 7 focuses on a possible role of host immune genetics. Through co-evolution, *M. tuberculosis* may have adapted to the immune system of particular human populations. I examined if specific gene polymorphisms of one host immune gene, *SLC11A1* (formerly called *NRAMP1*), which has been linked with TB in many different settings, were more common in patients infected with Beijing genotype strains.

Finally, in chapter 8 I performed a literature review on all possible factors that may contribute to the global spread of *M. tuberculosis* Beijing strains.

Chapter 9 contains the summary and general discussion of the main findings of this thesis. An effort is made to look comprehensively at the mechanism underlying the global emergence of *M. tuberculosis* Beijing genotype strains. Hopefully, this will help TB control in Indonesia and elsewhere.

References


Chapter 2

Application of spoligotyping to noncultured *Mycobacterium tuberculosis* bacteria requires an optimized approach

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*Journal of Clinical Microbiology, 2003, 41:11, 5350-5351*
In a recent issue we read with interest the contribution of Zink et al. (1) on the characterization of *Mycobacterium tuberculosis* complex DNA in Egyptian mummies by spoligotyping (spacer-oligo-typing).

Compared with other PCR-based methods that combine detection and typing of such DNA, spoligotyping is more sensitive, because it targets the direct repeats (DRs) present in multiple (sometimes up to 60) copies in the genomic DR locus of *M. tuberculosis* complex bacteria. The well-conserved 36-bp DRs are interspersed with nonrepetitive spacer sequences of 34 to 41 bp in length (2-4). The variation in the presence of these spacer sequences among strains of the *M. tuberculosis* complex allows for the genotyping of mycobacterial DNA directly isolated from clinical samples without the need to culture these bacteria.

Kamerbeek et al. described spoligotyping for use with purified DNA from cultured strains (2). However, when this method was used to target extracted DNA from *M. tuberculosis* complex bacteria in clinical samples, some of the spacers showed weak hybridization signals. We detected no hybridization signals with a few clinical samples. This is proven by the comparison of the spoligotyping patterns produced from bacteria on a Ziehl-Neelsen (ZN) slide (in dilution of 1:2, 1:4, and 1:8) with those seen with the method described by van der Zanden et al (3). (see Fig. 1). Therefore, we optimized our PCR mixture for direct spoligotyping of *M. tuberculosis* complex DNA in clinical samples by using a concentration of 3.0 mM instead of 7.0 mM MgCl₂, 15 mM instead of 5 mM Tris-HCl (pH 9.0), and 20 to 50 pmol of primer in PCR. The use of this adjusted protocol yielded a complete spoligotyping pattern for bacteria in clinical samples as compared to that seen with cultured bacteria (3).

We also successfully used the optimized spoligotyping on bacteria from paraffin wax-embedded tissues (4-6) and from the mummified remains of humans found in an 18th century Hungarian crypt (7). To our surprise, in the current study of Zink et al. (1) the nonoptimized protocol of Kamerbeek et al. (2) was applied to study samples from Egyptian mummies without any modification of the PCR reagents. Hence, in the spoligotyping patterns no hybridization with spacers 2, 14, and 39 was found. We therefore again wish to emphasize that for a reliable and reproducible application of spoligotyping to *M. tuberculosis* complex bacteria in clinical samples, the optimized protocol (3, 7) should be used.
Figure 1. Comparison of spoligotyping patterns after the application of spoligotyping directly to ZN-stained slides by using the protocols of Kamerbeek et al. (2) and van der Zanden et al. (3). The extracted DNA of the ZN slides was used in the dilutions 1:2, 1:4, and 1:8 in the spoligotyping.

References

Chapter 3

The population structure of *Mycobacterium tuberculosis* differs significantly on two Indonesian islands

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Abstract
Comparison of *Mycobacterium tuberculosis* genotype distribution in different areas might help to find determinants of the emergence of certain genotypes, like the Beijing family. In this study, *M. tuberculosis* isolates originating from patients from two Indonesian islands were genotyped, and possible associations with patients’ characteristics and drug resistance were explored.

A high degree of genetic diversity was observed among the *M. tuberculosis* strains and a significant difference was found in the geographical distribution of genotype families. The predominant Beijing genotype family was isolated from 268 of 813 patients from West-Java (33.0%) versus 12 of 84 patients from Timor (14.3%, p=0.002). Family F (EAI, 33.3%) and Family D (LAM, 20.0%) were more prevalent in Timor. No significant associations were found between genotype families and age, BCG vaccination, previous treatment, disease localization or drug resistance. Possible explanations for the differences in the geographical distribution of the *M. tuberculosis* genotypes are discussed.

Introduction
The introduction of DNA fingerprint methods in the early 1990s has greatly improved the possibilities to examine transmission of tuberculosis (TB) and the phylogeny of *M. tuberculosis* (1). The available genetic fingerprinting methods have different characteristics and applicability. Especially analysis of large chromosomal deletions (2), and single nucleotide polymorphisms can facilitate meaningful exploration of the population structure of the *M. tuberculosis* complex. However, these methods imply sophisticated and costly techniques. More widespread is the use of spoligotyping to examine the strain diversity of *M. tuberculosis* in given areas (1,3). With this method the presence of 43 spacers in the direct repeat (DR) region of *M. tuberculosis* complex can be detected. The loss of spacers in the DR region seems to be in line with the evolutionary development of the *M. tuberculosis* complex lineages and therefore, this currently appears to be the simplest approach to study the population structure of *M. tuberculosis* complex strains (4). Because a high number of spoligotype patterns have been added to a central database, many *M. tuberculosis* genotype families could be identified in the last 15 years, including the Beijing, the East African-Indian (EAI), the Haarlem (H), the Latin American and Mediterranean (LAM), and the Central Asian (CAS) genotype families (3).

One of the best studied and most widespread evolutionary lineages of *M. tuberculosis* is the Beijing genotype family which was first found in China and has been reported worldwide (5,6). The emergence of Beijing genotype strains suggests that they may have a selective advantage over other *M. tuberculosis*
strains. Indeed, studies in animal models have shown enhanced virulence and distinctive histo-pathology following infection with *M. tuberculosis* Beijing genotype strains (7,8). Extrapolating from this, emergence of Beijing and other genotype families may be the consequence of the application of two major measures against tuberculosis in last century: BCG vaccination and anti-tuberculosis treatment (1). Possibly, BCG-vaccination is less protective against (more virulent) Beijing genotype strains but so far, this has not been proven (9). Similarly, anti-TB treatment might be less effective in eradicating Beijing strains. Indeed, in one study failure of TB-treatment and subsequent relapse were more common in patients infected with Beijing strains (10). Studies examining a relationship between the Beijing genotype and drug-resistance have met with major differences in wide spread geographic areas, as systematically reviewed (5).

After India and China, Indonesia has the third highest TB case load in the world, with an estimated 525,000 new cases and an estimated 90,000 deaths per year (11). So far, only one molecular epidemiological study on tuberculosis was performed in Indonesia, in 1998 (12). Among 94 tuberculosis patients, all from the capital city Jakarta, 32.4% were infected with Beijing genotype strains. The Indonesian archipelago consists of 17,504 islands, inhabited by many different ethnic groups. Comparison of the distribution of *M. tuberculosis* genotypes in different areas or different human populations and study of the patients’ characteristics might help to find determinants of the emergence of particular genotype families. Therefore, we have spoligotyped *M. tuberculosis* isolates from a large cohort of patients from two Indonesian islands; West Java and Timor. Furthermore, we have explored the distribution of *M. tuberculosis* genotype families and analyzed the possible association of these genotypes with age, sex, BCG-vaccination status, previous TB treatment, disease localization and drug-resistance.

**Material and Methods**

**Study population**

In Indonesia, *M. tuberculosis* culture is not routinely performed, and there is no national archiving of isolates. Therefore, we prospectively collected isolates from well-characterized patients in two different clinical studies. In West Java, from January 2001 through December 2006, consecutive patients over 16 years of age with microscopically proven pulmonary tuberculosis were included in two outpatient clinics and two hospitals in Jakarta and Bandung (West Java), as part of a large case-control study examining host susceptibility to tuberculosis (13). The diagnosis of tuberculosis was based on clinical presentation, chest X-ray examination, microscopic detection of acid-fast bacilli by Ziehl-Neelsen stained sputum smear, and culture of *M. tuberculosis* on 3% Ogawa medium. As part of
the same study, a smaller cohort of microbiologically proven extra-pulmonary tuberculosis cases was included. All cultured *M. tuberculosis* isolates from patients with pulmonary (PTB; n=740) and extra-pulmonary (EPTB; n=73) disease were used for the current study. In addition, in West Timor, as part of a study on micronutrient supplementation in TB, 84 cultured isolates of pulmonary tuberculosis patients were included (Pakasi et al., in preparation). Of note; these were the first isolates ever cultured in this less-developed part of Indonesia. From all patients, age, history of previous TB treatment, the presence of a BCG-scar on the left deltoid muscle (as an approximation of BCG-vaccination status) and disease localization were recorded.

**Spoligotyping**
DNA was extracted by bringing two loops of bacterial mass from a *M. tuberculosis* culture in saline and subsequent heating at 95°C for 5 min. Spoligotyping was performed using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands). The presence or absence of 43 spacers in the DR region of isolates of *M. tuberculosis* was detected as follows: the DR region was amplified by primers, one of which was biotinylated; the amplified products were reversehybridized to spacer sequence oligonucleotide probes immobilized on a Biodyne C membrane; and detection of spacer sequences was achieved with peroxidase-labeled streptavidin and enhanced chemiluminescence (14,15). Spoligotyping was done at the Hasan Sadikin Hospital, Bandung, Indonesia. For quality control, spoligotyping of 10% of the isolates, and all isolates lacking hybridization, was repeated at Gelre Hospital, Apeldoorn, the Netherlands.

**Phylogenetic reconstruction**
The spoligotyping results were recorded in octal and binary formats in an Excel spreadsheet and compared to the international SpolDB4.0 database (3). The phylogenetic analysis was done using the Bionumerics software (Applied Maths, Sint-Maartens-Latem, Belgium). Spoligotype patterns were imported into Bionumerics as a character type, similarities between the patterns were calculated by using the categorical coefficient and dendrograms were prepared by using UPGMA.

**Drug susceptibility testing**
Drug susceptibility testing (DST) was performed on cultured isolates using an absolute concentration method on 7H10 Middlebrook agar in 25-well plates with supranational control by the National Mycobacteria Reference Laboratory at the RIVM in Bilthoven, The Netherlands. In the Netherlands, this method has shown accuracy between 96 and 100% in 10 rounds of WHO/IUATLD proficiency testing (16). Approximately 10% of isolates from the current study were retested at the supranational reference laboratory in The Netherlands.
Data-analysis and statistics
Spoligotype patterns were correlated with patient’s age, sex and BCG vaccination status (presence or absence of BCG scar), previous treatment, resistance to INH, rifampicine and both drugs (multidrug resistant TB). Differences between groups were statistically examined using non-parametric tests for continuous variables and Fisher’s $\chi^2$ test. To avoid possible confounding by geographical area, the above associations were examined separately for Java and Timor.

Results
Distribution of $M. tuberculosis$ genotype families
Eighty-four $M. tuberculosis$ isolates from Timor and 813 isolates from West Java were collected. Among the isolates from West Java, 740 were isolated from pulmonary TB patients and 73 from patients with extrapulmonary TB. The latter category consisted of meningitis ($n=42$), lymphadenitis ($n=9$), pleuritis ($n=13$), and other ($n=9$).

A high degree of genetic diversity among the $M. tuberculosis$ isolates was observed (Table 1). On the basis of the similarity of their spoligotype patterns, 739 of the 897 isolates were grouped into nine major spoligotype groups of more than 20 isolates. These nine major spoligotype groups were designated family A to family I. Spoligotype patterns outside of those nine major groups were designated ‘other’. The largest spoligotype group comprised the Beijing genotype (family I) and contained 280 strains (31.2%). The second largest group was family B (T1), which consisted of 100 isolates (11.1%). Families E and A, both representing the Haarlem genotype, contained 82 (9.1%) isolates. In this study 198 patterns were designated ‘orphan’ patterns, because they had no shared-type pattern in the international spoligo database SpolDB4 (Figure 1). No unique Indonesian strains were identified.

The distribution of genotypes showed little variation over time. From 2000 to 2006, between 28.9% and 32.7% of the strains belonged to the Beijing genotype family, with no obvious trend in the number of cases in time. As the distribution of genotypes in outpatient clinics and hospitals in Jakarta and Bandung was highly similar, those data were combined (‘West Java’). When the spoligotype patterns originating from West Java were compared to those from Timor, there appeared to be a significant difference in the distribution of genotype families. In West Java $M. tuberculosis$ Beijing genotype strains were the most predominant (33.0%, 95% CI 29.7-36.2%), but in Timor, Beijing strains were much less common (14.3%, 95% CI 6.8-21.8%; $p=0.002$). On the other hand, Family F (EAI) and Family D (LAM) were highly prevalent in Timor (33.3% respectively 20.0%) but uncommon in West Java (6.2% respectively 8.7%) (Figure 2). All these differences were statistically significant.
Association of genotype families with patient characteristics

We compared the patient characteristics of different *M. tuberculosis* genotype families, including sex, age, BCG vaccination, previous treatment, and disease localization (PTB vs EPTB). Because of the strong geographic differences in the distribution of genotypes, data from West Java and Timor were analyzed separately. For the 813 patients included in West Java, the results are depicted in Table 2. The various genotype families were associated with slight differences in patient characteristics, none of which were statistically significant. BCG scar as an approximation of BCG vaccination was only found in 28.7% of patients. The smallest percentage of those who had BCG scar was found in patients infected with strains from family C (T) (16.3%) and the highest was found in patients infected with strains from family A (H) (46.3%), but these differences did not reach statistical significance. Similarly, different genotype families showed slightly different age distribution, percentage of extra-pulmonary localization and previous treatment, but none of these were statistically significant (Table 2).

To relate the *M. tuberculosis* genotype and patients characteristics in Timor (not included in Table 2) we compared the two largest families there; family F (EAI) (33.3%) and family D (LAM) (22.0%), with the remaining group including all strains of other genotypes (46.7%). The median age of patients infected in the second largest genotype family (Family D/LAM), was significantly different than the median age of the other families (23 years vs. 29 and 32, p=0.010). No significant differences between genotype families were found among other patient characteristics. When the twelve (14.3%) patients infected with a Beijing genotype were analyzed separately, they appeared to be older than the patients infected with other strains (median 46 years, IQR: 24.3-57.5), but this was not statistically significant.

Association of genotype families with drug resistance

Drug resistance patterns were available for 694 isolates, 620 from West Java, and 74 from Timor. Among the isolates of previously treated patients (n=77), 25.4% were INH resistant, 31.2% were rifampicin resistant, and 17.9% were MDR. Among newly treated patients (n=617) these rates were 7.5%, 6% and 2.6%, respectively. The various genotype families showed slightly different rates of drug resistance. Resistance to INH ranged from 2.7% to 10%, and resistance to rifampicin ranged from 2.7% to 11.2%. Because the number of drug resistant isolates per genotype family was too small no meaningful statistical analysis could be done on those differences between the nine genotype families.

Several previous studies have reported an association between the Beijing genotype and drug-resistance, drug resistance patterns of 209 Beijing isolates (30.1%) and 485 non-Beijing isolates (69.9%) were not significantly different (table
2). Also when strains isolated from Java were analyzed separately, no association was found between the Beijing genotype and drug-resistance.

### Table 1. Spoligotype diversity and spoligotype family designation of 897 *M. tuberculosis* isolates from West Java and Timor.

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<tr>
<th>Spoligo family (SpoIDB4)</th>
<th>Spoligo family designation this study</th>
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<td>LAM3</td>
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<td>MANU2</td>
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<tr>
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<td>S</td>
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<tr>
<td>T2</td>
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<tr>
<td>T3</td>
<td></td>
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<tr>
<td>T5_RUS1</td>
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<tr>
<td>U</td>
<td></td>
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<tr>
<td>U (LIKELYH3)</td>
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<td>U (LIKELYS)</td>
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Total: 41 100 43 90 41 74 41 29 280 158 897 307
### Table 2. *M. tuberculosis* genotype families and associated patient characteristics in West Java

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<thead>
<tr>
<th>Spoligotype family</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>Other</th>
<th>Total</th>
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<tr>
<td>Spoligotype family SpolDB 4</td>
<td>(H)</td>
<td>(T)</td>
<td>(T)</td>
<td>(LAM)</td>
<td>(H)</td>
<td>(EAI)</td>
<td>(U likely H3)</td>
<td>(U likely S)</td>
<td>(Beijing)</td>
<td>(U likely H3)</td>
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</tr>
<tr>
<td>41 (5%)</td>
<td>98 (12.1%)</td>
<td>43 (5.3%)</td>
<td>71 (8.7%)</td>
<td>40 (4.9%)</td>
<td>50 (6.2%)</td>
<td>37 (4.4%)</td>
<td>27 (3.3%)</td>
<td>368 (43.3%)</td>
<td>138 (17.3%)</td>
<td>813 (100%)</td>
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<tr>
<td>Age (yrs)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Median</td>
<td>26.0</td>
<td>28.5</td>
<td>30.0</td>
<td>26.0</td>
<td>27.0</td>
<td>34.5</td>
<td>27.0</td>
<td>27.0</td>
<td>36.0</td>
<td>35.0</td>
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<tr>
<td>IQR</td>
<td>20.5-36.0</td>
<td>23.0-42.0</td>
<td>24.0-46.0</td>
<td>25.0-36.0</td>
<td>21.3-41.8</td>
<td>24.0-48.3</td>
<td>22.5-48.0</td>
<td>20.0-41.0</td>
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<td>Sex (male, %)</td>
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<td>BCG-scar (%)*</td>
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<td>24.5</td>
<td>16.3</td>
<td>25.6</td>
<td>30.0</td>
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<td>22.2</td>
<td>28.0</td>
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<tr>
<td>Previous TB-treatment (%)</td>
<td>12.2</td>
<td>16.3</td>
<td>4.7</td>
<td>8.5</td>
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<td>20.0</td>
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<td>extrapulmonary (%)</td>
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<td>9.2</td>
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<td>25.9</td>
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<td>pulmonary (%)</td>
<td>97.6</td>
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<td>84.1</td>
<td>89.1</td>
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<td>Drug resistance (%)**</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Isoniazide (H+I)</td>
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<td>14.3</td>
<td>11.6</td>
<td>7.0</td>
<td>17.5</td>
<td>14.0</td>
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<td>11.1</td>
<td>13.1</td>
<td>9.4</td>
<td>12.3</td>
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<tr>
<td>Isoniazide (Only R)</td>
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<td>7.1</td>
<td>7.0</td>
<td>4.2</td>
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<td>10.0</td>
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<tr>
<td>Rifampicin</td>
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<td>11.2</td>
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<td>10.0</td>
<td>8.0</td>
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<td>7.4</td>
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<tr>
<td>MDR</td>
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<td>4.1</td>
<td>2.3</td>
<td>2.8</td>
<td>5.0</td>
<td>6.0</td>
<td>0.0</td>
<td>3.7</td>
<td>3.7</td>
<td>4.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* BCG-scar was evaluated in 565 patients; ** DST was successful in 694 isolates
Figure 1
Figure 1 - continued

Figure 1  Dendrogram (left) showing the similarity among the 198 orphan spoligotype patterns (middle) found in this study. On the right the number of isolates of a particular spoligotype is indicated.
Discussion
This is the second study on the molecular epidemiology of tuberculosis in Indonesia, involving the two most densely populated cities in West Java and a more rural area on Timor at the eastern end of the archipelago. Consecutive microscopically proven tuberculosis patients from a large cohort study in various clinics in 2001-2006 were included, and patients were carefully characterized and followed prospectively. Because the isolates only came from three areas, the study is not representative for Indonesia as a whole. However, the four clinics in West Java showed a very similar M. tuberculosis genotype family distribution, suggesting that these data are indeed representative for this area.

A high genetic diversity among M. tuberculosis isolates was found in Indonesia: nine major M. tuberculosis genotypes were identified. This high genetic M. tuberculosis strain diversity is in contrast with studies from other higher prevalence areas, including e.g. Vietnam, where only two major genotype families were found: Beijing and EAI (10). The M. tuberculosis Beijing genotype family was the most prevalent genotype family in Indonesia (31.2%), and the proportion of Beijing strains was stable during the study period and similar to the prevalence...
recorded in a previous, smaller study (32.4%) (12). In contrast to what has been reported in others studies (9,10,17), no association was found between the Beijing genotype and age, previous treatment and BCG vaccination. As demonstrated previously in other studies from Asia, no clear association was found between drug resistance and Beijing strains as reported in e.g. the Former USSR republics, Cuba, South Africa and Vietnam (6).

Interestingly, a strong difference was found in the population structure of \( M. \) tuberculosis in West Java and Timor. The Beijing genotype family was found in 33.0% of patients in Java, versus 14.3% of patients in Timor. This is in line with a recent observation in Vietnam, where in Ho Chi Minh City a density of 50% was notified for Beijing genotype strains, but in the Mekong Delta only 30% (unpublished observation). This suggests that transmission of Beijing genotype strains may benefit from highly dense populations. Inversely, in Timor, the EAI and LAM genotype families were predominant, while these genotypes were uncommon in Java. One can only hypothesize about the explanation for this difference. First, this difference may be due to a “founder effect”, with a higher chance of finding a particular genotype family closer to where it originated. Secondly, it may also indicate that particular mycobacterial lineages have adapted to particular human populations. This concept of genetic host-pathogen compatibility is supported by data showing preferential spread of particular lineages in patient populations from the same area, rather than from other areas (18,19). So far, no studies have been reported making direct associations between genetic characteristics of tuberculosis patients and their (own) mycobacterial isolate.

Finally, the predominance of certain genotype families, particularly the Beijing genotype family, might also be explained by other mechanisms, such as higher transmission rate or ‘escape’ from BCG-vaccination. Our results do not support these hypotheses, as no significant associations were found between particular genotype families and patient characteristics, especially age and BCG-vaccination status. However, the coverage of BCG vaccination is very high in Indonesia. Hence, transmission from vaccinated to none-vaccinated subjects is so frequent, that differences in the population of strains in both groups will be diluted easily.

Several studies, like one in Vietnam have reported a lower age of patients infected with Beijing strains (9), suggesting more recent transmission of Beijing strains. A second study from a different area in Vietnam has found a similar relationship between age and genotype (Buu, submitted), but, like others (17,20) we did not find this association. In fact, among patients from Timor, those infected with a Beijing strain were older, although the number was small. We also did not find an association with BCG-vaccination, like one study in Vietnam (9). The difference between Indonesia and Vietnam may be explained by the existence of different
evolutionary lineages of the Beijing genotype family, circulating in different geographic areas as has been demonstrated previously (21).

In Indonesia, drug susceptibility testing of *M. tuberculosis* is a matter of concern. Indonesia is not included in international surveillance of drug resistance (22) and there is no national surveillance, or quality control system. We have previously compared the quality of conventional (proportional) DST, and the 25-well DST method (16) in Indonesia, and it was shown that the 25-well DST method used in this study showed a better performance on WHO reference strains (Alisjahbana et al., submitted for publication). However, the high rates of drug resistance which were found in this study are in contrast with the only recent peer-reviewed data on drug resistance in Indonesia, and rates in neighboring countries (23). Because most of the isolates in this study came from clinics, care should be taken with the interpretation of the high drug resistance rate observed. Drug resistance rates in a more nationally representative DST-survey may not be as high. It is clear that there is an urgent need for widespread implementation of quality-assured drug susceptibility testing in Indonesia. Because of the limited number of strains belonging to certain genotype families, a possible relationship between particular genotypes and drug resistance cannot be excluded.

In conclusion, this molecular epidemiological study in Indonesia shows a considerable degree of heterogeneity among *M. tuberculosis* isolates, and a significant difference in population structure at the different geographical study sites. A nationwide survey can provide more detail on the distribution of different genotype families in Indonesia, and association of host and mycobacterial genetics may help to establish if differences in population structure of *M. tuberculosis* are caused by evolutionary adaptation of particular mycobacterial lineages to certain human populations.

**Acknowledgement**

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Reference


Chapter 4

*Mycobacterium tuberculosis* Beijing genotype in Indonesia is associated with drug resistance gene mutations in *katG*, *rpoB* and *embB*

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*Submitted*
Abstract
Mycobacterium tuberculosis Beijing genotype strains are highly predominant in most parts of Asia suggesting they have a selective advantage such as drug resistance. We examined the possible association between the Beijing genotype and drug resistance gene mutations among isolates from Indonesian patients.

We performed spoligotyping, sequencing of the rpoB-hotspot region and PCR for mutations in katG codon 315 and embB codon 306 on phenotypically drug-resistant and -sensitive patient isolates.

Among 108 Beijing and 203 non-Beijing M. tuberculosis isolates, rpoB-mutations were found in 33.3% respectively 18.7% (p = 0.004), the katG315 mutation in 18.5% vs. 12.8% (p = 0.177), and the embB306 mutation in 16.5% vs. 6.1% (p = 0.006). Among newly treated patients, rpoB-mutations were found in 19.7% of Beijing vs. 6.7% of non-Beijing strains (p = 0.005).

The higher rate of mutations in rpoB, katG315 and embB306 among M. tuberculosis Beijing strains provides further evidence that drug resistance may contribute to the global predominance of Beijing strains. The strong association of Beijing and rpoB-mutations among new cases of TB reflects considerable transmission of resistant Beijing strains in Indonesia.

Introduction
Epidemiological genotyping studies of M. tuberculosis isolates from clinical patients have shown a predominance of particular genotypic families, like Beijing, CAS, EAI, Haarlem and LAM (1). The Beijing genotype is especially predominant in Asia, with prevalence ranging from 30% to more than 80% in different areas (2). Its wide distribution suggests the Beijing genotype family may have a selective advantage. First, Beijing genotype strains might be more easily transmitted. Alternatively, they might be more virulent, causing a higher proportion of infected individuals to develop active tuberculosis, or a lower proportion to be protected by BCG-vaccination. Studies exploring these hypotheses have shown conflicting results. Animal studies indeed suggest that Beijing strains are more virulent (3), but epidemiological studies examining associations between genotype and BCG-vaccination (4-6) disease severity (6, 7) or radiological appearance (6-8) have shown contrasting results. Finally, Beijing genotype strains might more readily become or remain drug-resistant, thereby hampering their eradication. A systematic review (including studies using phenotypic drug susceptibility testing) found no clear association between the Beijing genotype and drug resistance (2). On the other hand, genotypic studies have reported a higher frequency of katG codon 315 mutations (responsible for 65 to 100% of resistance to isoniazid) among Beijing genotype strains (9-11). As a possible explanation for this
phenomenon, Beijing strains were found to have more mutations in DNA-repair genes, suggesting they may more easily develop mutations which lead to drug resistance (12), although this not been found in vitro (13).

So far, it is uncertain if the Beijing genotype is associated with other drug resistance mutations besides katG315. We have addressed this question in Indonesia, which has the third highest tuberculosis case-load worldwide (14). We characterized a collection of *M. tuberculosis* patient isolates to see if the Beijing genotype, found in 33% of Indonesian tuberculosis patients, (15) had a higher rate of mutations in katG315, the rpoB-hotspot region, which is responsible for >90% of resistance to rifampicin (10, 11, 16), and embB306, which accounts for resistance to ethambutol, and which may predispose *M. tuberculosis* to develop resistance to other antibiotics (17-20).

**Methods**

*Mycobacterium tuberculosis* isolates

Genotyping and drug resistance testing (DST) of *M. tuberculosis* is not routinely performed in Indonesia, and there is no national or supranational quality control system. We collected patient isolates from four laboratories in Jakarta and Bandung that do perform DST for INH and rifampicin. Primary culture in these laboratories is done using solid medium (Ogawa 3%), and DST is done using a proportional method, and more recently in two laboratories using an absolute concentration method on 7H10 Middlebrook agar in 25-well plates (21).

Spoligotyping

Spoligotyping was performed on a total of 812 *Mycobacterium* isolates from West-Java (15). DNA was extracted from isolates by bringing two loops of bacterial mass in saline and subsequent heating at 95°C for 5 min. Spoligotyping was performed using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands). The presence or absence of 43 spacers in the direct repeat (DR) region of isolates of *M. tuberculosis* was detected as follows: the DR region was amplified by primers, one of which was biotinylated; the amplified products were reverse-hybridized to spacer sequence oligonucleotide probes immobilized on a Biodyne C membrane. Detection of spacer sequences was achieved with peroxidase-labeled streptavidin and enhanced chemiluminescence. Spoligotyping was done at Hasan Sadikin Hospital, Bandung, Indonesia. For quality control, spoligotyping was repeated in the Netherlands for 10% of isolates, and for all isolates lacking hybridization.
Sequencing of \textit{rpoB}-hotspot region

Three hundred and twenty five spoligotyped isolates were \textit{rpoB} sequenced, 116 phenotypically rifampicin resistant strains, 178 random rifampicin sensitive isolates, and 31 strains for which sensitivity testing was unsuccessful. Sequencing of \textit{rpoB} was done using 3100-Avant sequencer (Applied Biosystems). Amplification of a 437 bp fragment containing the \textit{rpoB} hotspot region was carried out in PTC 200 thermocycler (Biozym) by \textit{rpoB}-F1 forward and \textit{rpoB}-R1 reverse primer (22). The Sequencing results were compared to \textit{rpoB}-hotspot wildtype using Bionumerics software (Applied Maths, St-Martin-Latern, Belgium). Sequence analysis (16S rDNA) blasting against the Genebank database showed that 14 isolates (4.3%), 13 of whom were phenotypically rifampicin resistant, were non-tuberculous mycobacteria, including \textit{Mycobacterium massiliense} (n=4), \textit{M. fortuitum} (2), \textit{M. abscessus} (2), \textit{M. asiaticum} (1), \textit{M. mageritense} (1), \textit{M. perigrinum} (2), \textit{M. chelonei} (1) and undetermined mycobacterial species (1). Spoligotyping of primary cultures from the same patients showed \textit{M. tuberculosis} patterns, so it was concluded that these 14 isolates were most likely culture contaminants. These isolates were excluded for further analysis, leaving a total number of 311 isolates.

Mutation analysis of \textit{katG315} and \textit{embB306}

DNA amplification/detection for \textit{katG315} was performed either on a ABI Prism$^\text{™}$ 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) or on a LightCycler480 (Roche Diagnostics, Indianapolis). For the ABI Prism$^\text{™}$ 7000, we amplified \textit{katG315} using a set of primers; forward primer (5’GGG CTT GGG CTG GAA GAG3’) and reverse primer (5’ACA ACA GTT TCC TGC AGA TCC TGT3’). Detection of \textit{katG315} wildtype was done by FAM-TAMRA labeled probe (5’CGCGATCACCAGCGGCATCG3’) and detection of \textit{katG315} mutation was done by VIC-TAMRA labeled probe (5’CGC GAT CAC CAC CGG CAT CG 3’). DNA amplification/detection was performed in 30μl reaction volume : template DNA, 400nM of each primer and 200nM of each probe. The PCR-program consisted of 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 seconds at 95°C, 1 min at 60°C. For the LightCycler480 we amplified \textit{katG315} using the same primers and the same probes except with other labels namely : the wildtype probe was labeled with FAM-BBQ and the mutant probe with Cyano500-BBQ. DNA amplification / detection was performed in 30μl reaction volume : 20μl Taqman Universal PCR Master Mix (Applied Biosystems) and 10μl template DNA, 400nM of each primer and 200nM of each probe. The PCR program consisted of 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 seconds at 95°C, 1 min at 60°C. The detection of the \textit{embB306} mutation was performed on the ABI Prism$^\text{™}$ 7000. The primers used for amplification were forward primer \textit{embB1} (5’-CGGCTTCCCGAGACCCAACCTG-3’) and reverse primer \textit{embB2} (5’-GCTGTACCGCATGGACCGCTC-3’). Detection of \textit{embB306} was done by a FAM-
TAMRA labelled LNA probe (5’FAM-CATGGCCCGAGTCGCC-TAMRA3’) and a control VIC-TAMRA labelled probe (5’VIC-GGCATGTCATCGGCGAAT-TAMRA3’). Instead of using a mutant probe a control probe was used. The wildtype (LNA) probe hybridizes if codon 306 contains no mutations. The control probe hybridizes to a specific piece of DNA without known mutations. The wildtype probe gives a signal when the strain contains no mutation on codon 306 and the control probe gives a signal if an amplification product with a mutation in codon 306 is present.

Data analysis and statistics
The frequency of gene mutations of *M. tuberculosis* Beijing and non-Beijing genotype isolates was compared using Pearson’s Chi-square. We considered P-values < 0.05 as significant. Odds ratios (OR) and 95% confidence intervals (CI) for associations between genotype and particular drug resistance gene mutations were calculated. Using regression analysis, corrections were made for possible confounding by previous treatment. Data were analyzed using the Statistical Package for Social Sciences (SPSS Inc, Chicago, IL version 12.01).

Results
Mutations in *rpoB*
Sequencing of *rpoB* showed mutations in 74 *M. tuberculosis* patient isolates. Mutations were mostly found in *rpoB* codon 531 (52.7%), codon 526 (31.1%), and codon 516 (8.1%), either as single or double mutations. The distribution of *rpoB*-mutations is shown in Table 1. Among 48 patients with a history of previous tuberculosis treatment, 19 (39.6%) were infected with *rpoB*-mutant strains, compared to 23 of 205 (11.2%) of newly treated patients (OR 5.18; 95% CI 2.52 – 10.68). From 103 phenotypically rifampicin-resistant strains *M. tuberculosis*, 42 (40.8%) showed no *rpoB*-mutation while from 177 phenotypically rifampicin-sensitive *M. tuberculosis*, 13 (7.3%) had a mutation in the *rpoB* gene. In line with the discrepancy between phenotypic and genotypic results, proficiency testing in 2004/2005 in the participating laboratories using 30 WHO strains yielded a sensitivity and specificity of 89.2% and 85.1% for INH, and 89.5% respectively 75.2% for rifampicin (B. Alisjahbana, personal communication).

Mutations in *katG315* and *embB306*
Analysis of *katG* codon 315 showed mutations in 46 isolates (14.8%). Mutations were found in 2 of 175 INH-sensitive isolates (1.1%) and 41 of 105 INH-resistant isolates (39.0%). Mutations in *katG315* were associated with previous treatment (OR 2.02; 95% CI 0.86 – 4.75), and also with mutations in *rpoB* (OR 7.41; 95% CI 3.78 – 14.50). Among 262 isolates, a total of 26 (8.4%) showed a mutation in *embB* codon 306. Mutations in *embB306* were more common among previously treated patients (OR 2.85; 95% CI 0.76 – 10.60), and strongly associated with
mutations in \( rpoB \) (OR 33.46; 95%CI 9.61 – 115.41), and \( katG315 \) (OR 5.45; 95% CI 2.28 – 13.03).

**Associations between genotypic drug resistance and spoligotype**

The genotype distribution in this study was in line with spoligotyping of 812 mycobacterial isolates from West-Java (15), showing a high genetic diversity, with nine different genotype families, and a prevalence of 34.7% of Beijing genotype strains. For all three genes examined, mutations were more common among \( M.\ tuberculosi s \) Beijing genotype strains than among other genotype strains (Table 2). Mutations in \( rpoB \) were found in 33.3% of \( M.\ tuberculosi s \) Beijing strains, compared with 18.7% of other genotype strains (OR 2.17; 95% CI 1.27 - 3.70; \( p = 0.004 \)). There was a difference in the distributions of specific \( rpoB \)-mutations between Beijing and non-Beijing strains (Table 1). The prevalence of mutations in \( rpoB \) codon 531 was lower in Beijing genotype than non-Beijing genotype strains (41.7% vs 63.2%), while the opposite was true for mutations in \( rpoB \) codon 526 (36.1% vs. 26.3%), although these differences were not statistically significant. The association between Beijing genotype and \( rpoB \)-mutations was not due to confounding by previous treatment. Among 205 newly treated patients (new cases), \( rpoB \)-mutations were found in 19.7% of patients infected with Beijing strains, versus 6.7% of patients infected with other genotype strains (OR 3.41; 95% CI 1.40 – 8.34; \( p = 0.005 \)).

Mutations in \( katG315 \) were more common among Beijing strains (Table 2), also when only new strains were examined (13.4% vs 8.7%), but neither of these associations was statistically significant. Mutations in \( embB306 \) were more common among Beijing genotype strains, and this was strongly significant (OR 3.06; 95% CI 1.33-7.05; \( p=0.006 \)) (Table 2).
Table 1. Drug resistance gene mutations in *M. tuberculosis* Beijing and other genotype strains.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Beijing genotype</th>
<th>other genotypes</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(n=108)</td>
<td>(n=203)</td>
<td>(n=311)</td>
</tr>
<tr>
<td><strong>rpoB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no mutation</td>
<td><strong>72 (66.7%)</strong></td>
<td><strong>165 (81.3%)</strong></td>
<td><strong>237 (76.2%)</strong></td>
</tr>
<tr>
<td>single mutation</td>
<td><strong>35 (32.4%)</strong></td>
<td><strong>35 (17.2%)</strong></td>
<td><strong>70 (22.5%)</strong></td>
</tr>
<tr>
<td>Gly→Lys</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Asp→Val</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ser→Leu</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>His→Arg</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>His→Leu</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>His→Asp</td>
<td>7</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>His→Tyr</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>His→Asn</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ser→Leu</td>
<td>12</td>
<td>19</td>
<td>31</td>
</tr>
<tr>
<td>Ser→Trp</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Pro→His</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>double-mutations</td>
<td>1 (1.0%)</td>
<td>3 (1.5%)</td>
<td>4 (1.3%)</td>
</tr>
<tr>
<td>Gly→His / His→Pro</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leu→Pro / Asp→Ala</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Deletion</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Leu→Met / Ser→Leu</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>katG315</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no mutation</td>
<td>88 (81.5%)</td>
<td>177 (87.2%)</td>
<td>265 (85.2%)</td>
</tr>
<tr>
<td>mutation (Ser→Thr)</td>
<td>20 (18.5%)</td>
<td>26 (12.8%)</td>
<td>46 (14.8%)</td>
</tr>
<tr>
<td>embB806</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no mutation</td>
<td>81 (83.5%)</td>
<td>155 (93.9%)</td>
<td>236 (90.1%)</td>
</tr>
<tr>
<td>mutation</td>
<td>16 (16.5%)</td>
<td>10 (6.1%)</td>
<td>26 (9.9%)</td>
</tr>
</tbody>
</table>

* PCR for *embB* was only performed in 97 Beijing and 165 other genotype strains
Table 2. Association between *M. tuberculosis* genotype and drug resistance gene mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Beijing genotype (n=108)</th>
<th>other genotypes (n=203)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td>36 / 108 (33.3%)</td>
<td>38 / 203 (18.7%)</td>
<td>2.17 (1.27-3.70)</td>
</tr>
<tr>
<td>katG315</td>
<td>20 / 108 (18.5%)</td>
<td>26 / 203 (12.8%)</td>
<td>1.55 (0.82-2.92)</td>
</tr>
<tr>
<td>embB306</td>
<td>16 / 97 (16.5%)</td>
<td>10 / 165 (6.1%)</td>
<td>3.06 (1.33-7.05)</td>
</tr>
</tbody>
</table>

Discussion

A higher frequency of drug resistance might contribute to the global predominance of the Beijing genotype family. Although a systematic review about the Beijing genotype found no association with phenotypic drug resistance (2), genotypic studies looking at *katG*315 did indeed report higher mutation rates among Beijing strains (11, 23, 24). For *rpoB* this is less clear, as studies comparing Beijing and other genotype strains have examined the distribution (exact genetic location), rather than the overall frequency of *rpoB*-mutations (9-11). In this study from Indonesia we report a higher rate of *rpoB*-mutations among Beijing strains, both among previously treated and newly diagnosed tuberculosis patients. Similarly, *katG*315 showed more mutations among Beijing strains, although this difference was smaller and did not reach statistical significance. Finally, for mutations in *embB*306, responsible for resistance to ethambutol, we found a very strong association with the Beijing genotype similar to a smaller study from Russia (25).

If Beijing genotype strains are indeed associated with drug resistance gene mutations, what could be the explanation? Firstly, it might be due to a founder effect, with a particular strain with certain genetic characteristics circulating in a specific area. However, the strong genetic variability of *M. tuberculosis* isolates in this area (15), collected from different sites over a 6-year period of time, argues against bias of our findings by one particular (resistant) cluster. Second, Beijing genotype strains might show a higher mutation frequency. It was previously found that Beijing strains have alterations in so-called ‘mutator genes’, resulting in a defective DNA-repair system allowing an increased mutation rate (12, 26). One might also hypothesize that specific characteristics of the cell-wall structure of Beijing strains lead to suboptimal intracellular concentrations of antituberculous drugs and acquisition of drug resistance (27-29). Both these hypotheses need further proof: in vitro, Beijing strains did not appear to acquire drug resistance more easily (13, 26).
A third possible explanation for our findings is that Beijing strains with drug resistance mutations are more viable. At least for \textit{rpoB}, acquisition of drug resistance seems to be associated with reduced fitness of \textit{M. tuberculosis} (30, 31). Beijing genotype strains may be more virulent than non-Beijing strains (32), and this might compensate for the loss of fitness associated with mutations in drug resistance genes. This hypothesis is supported by differences we and others have found in the distribution of specific mutations in \textit{rpoB} (11). The most common mutation, Ser531Leu, which seems to have very little effect on fitness (30, 31), had a higher prevalence among non-Beijing strains, while other mutations (including those in codon 526), which seem to have a higher fitness ‘cost’ were more common among Beijing strains. Similar to a study among South African children, we found a stronger association of Beijing and \textit{rpoB}-mutations among newly diagnosed patients than among previously treated patients, suggesting easier transmission (more ‘fitness’) of resistant Beijing strains (33).

Mutations in \textit{embB}306 may account for ethambutol resistance in \textit{M. tuberculosis} (34, 35) but may also correlate with multiple drug resistance (17, 20, 34, 36). Also in our study, mutations in \textit{embB}306 strongly correlated with mutations in \textit{rpoB} and \textit{katG}315. As our study is cross-sectional, we cannot determine a causal relationship between mutations in \textit{embB}306, \textit{rpoB} and \textit{katG}315. Obviously, resistance to one drug increases the risk of acquiring resistance to another drug. Longitudinal studies with consecutive isolates from single patients may help to determine which mutations generally occur first. Irrespective of the underlying mechanism our study confirms that mutations in \textit{embB}306 are highly predictive of multidrug resistance.

The high rates of drug-resistance in this study should not be extrapolated to Indonesia, as we purposely selected resistant strains. However, our study does confirm the need for quality-assured drug susceptibility testing (DST) in this country. Established methods for DST (for at least rifampicin) may be inaccurate in this setting. DST is scarcely performed in Indonesia, mostly using the Canetti-method which dates back to 1955. Indonesia is not included in international surveillance of drug resistance (35), and there is no national surveillance, or quality control system yet. \textit{RpoB}-sequencing in our study confirmed that such a quality control system is urgently needed. Our sequencing results also showed the unexpected occurrence of atypical mycobacteria among cases which were considered rifampicin-resistant tuberculosis. We suspect that contamination like this may happen more often in low-resource settings, obviously affecting the reliability of DST.

Genotypic testing might play a role in improving routine susceptibility testing. Sequencing of \textit{rpoB} is no ideal option for Indonesia but a multiplex PCR combining the three most common targets would cover >90% of the resistance mutations. In
close collaboration with the WHO / TDR, a manual nucleic acid amplification test including these targets is being tested in low-resource settings (www.finddiagnostics.org).

In conclusion, we have examined 311 well-characterized M. tuberculosis strains from West-Java (population 40 million). Our results showed a higher rate of drug resistance mutations among Beijing genotype strains, which make up one third of all strains in this area. This suggests that, at least in part, the predominance of Beijing strains in Indonesia may be related to drug resistance. Unlike most other studies, we have been able to correct our results for previous treatment, which is important, as recurrent tuberculosis is obviously associated with drug resistance, but possibly also with the Beijing genotype (37, 38). The strong association of Beijing and rpoB-mutations among new cases of tuberculosis reflects considerable transmission of resistant Beijing strains in this setting. Our data also underline the need for implementation of quality-controlled drug resistance testing in Indonesia. Further study is needed to ascertain the underlying mechanisms for the associations found as well as its possible implications for tuberculosis control in Indonesia and elsewhere.

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None of the authors has any conflict of interest.

References


Chapter 5

*Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia

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Abstract
Animal studies have shown that strains of the globally emerging *Mycobacterium tuberculosis* Beijing genotype are more virulent compared to other strains. We examined if the Beijing genotype is associated with severity of tuberculosis and treatment failure in Indonesia. Among consecutive patients, the *M. tuberculosis* Beijing genotype was responsible for 33.4% of 818 pulmonary tuberculosis cases and 28.4% of 134 tuberculous meningitis cases (NS). No clear association was found between Beijing genotype and disease presentation or chest X-ray abnormalities. Patients infected with Beijing genotype strains more often had a positive sputum culture after six months treatment (RR:1.95; CI 95%:1.25-3.02), and this was not caused by differences in drug resistance. These data suggest that *M. tuberculosis* Beijing genotype strains have a higher capacity to withstand tuberculosis treatment, even in the absence of drug-resistance. Further study is needed to explore the underlying mechanisms as well as the possible implications for tuberculosis treatment.

Introduction
Molecular epidemiology has helped to reveal the evolutionary origins and phylogenetics of *Mycobacterium tuberculosis*. Evidence indicates that distinct *M. tuberculosis* lineages (genotypes) differ in terms of transmissibility, virulence, drug resistance, and treatment response. One of the best studied and most widespread evolutionary lineages of *M. tuberculosis* is the Beijing genotype family strains (1-3). This genotype may have originated from the Beijing region of China, and appears to have spread and become established as a predominant and endemic *M. tuberculosis* genotype in East and Southeast Asia, but also South Africa and the former Soviet Union states (1, 4). All *M. tuberculosis* lineages have recently expanded, but the Beijing family has had by far the largest population increase (5). This suggests that Beijing strains may have a selective advantage over other *M. tuberculosis* strains such as enhanced transmissibility, a lower protective effect of BCG-vaccination, reduced sensitivity to antituberculous drugs, or increased virulence. With regard to virulence, Beijing strains have indeed shown increased in vitro outgrowth in human monocytes (6) and macrophages (7) and increased mycobacterial outgrowth, distinctive histopathology and higher mortality in murine models (8, 9).

However, less is known about a possible correlation between causative *M. tuberculosis* genotypes like the Beijing family and clinical phenotype of tuberculosis, and different patient studies have met with conflicting results. For instance, in Russia and Vietnam, Beijing strains were associated with more severe chest X-ray abnormalities (10, 11), while in Singapore the opposite (12), and in the Netherlands no relationship was found (13). A higher virulence of *M. tuberculosis*
Beijing genotype strains might also result in more dissemination of mycobacteria leading to extrapulmonary tuberculosis. Indeed, a study conducted in Arkansas found that the Beijing genotype was associated with extrapulmonary TB (14). However, a study from South Africa failed to find such a relationship (15). Finally, the successful spread of Beijing genotype strains might also be caused by more treatment failures and relapse of tuberculosis. Indeed, patients in Vietnam showed a nearly three-fold higher rate of relapse and treatment failure when infected with *M. tuberculosis* Beijing genotype strains (16), and a higher rate of previous treatment was found among patients infected with Beijing strains in Russia (10). However, as the Beijing genotype has shown a relationship with drug-resistance in a number of studies (1) it remains uncertain whether the higher rate of treatment failure and relapse is due to intrinsic properties of the Beijing genotype perse, or to its association with drug-resistance.

As pointed out above, the degree to which the *M. tuberculosis* genotype influences the clinical phenotype of tuberculosis remains poorly understood. Few studies have combined detailed patient characteristics and treatment results with genotyping of *M. tuberculosis* isolates from sufficient numbers of patients, and so far only one longitudinal (retrospective) study has been reported (17). We have previously spoligotyped a large number of *M. tuberculosis* patient isolates from Indonesia, showing that 33% belonged to the Beijing genotype (18). In this prospective cohort study we have examined if Indonesian patients infected with Beijing genotype strains show a more severe clinical presentation, more disseminated tuberculosis, or more treatment failure compared to patients with other (non-Beijing) genotype strains. With relatively high number of patients we were able to examine drug resistance as a separate variable in multivariate analysis.

**Methods**

**Setting and patients**

In the context of a study exploring the role of host susceptibility to tuberculosis, consecutive new pulmonary TB patients above 15 years of age were included from October 2000 until December 2005 in three outpatient clinics in Jakarta and Bandung (19). Written informed consent was obtained from all subjects, and the study was approved by the ethical committee of the Faculty of Medicine, University of Indonesia, Jakarta and the Faculty of Medicine, Padjadjaran University, Hasan Sadikin Hospital, Bandung. Tuberculosis was diagnosed based on clinical presentation and chest X-ray examination, confirmed by positive microscopic detection of acid-fast bacilli (AFB). Tuberculosis treatment consisted of a standard regimen of daily rifampicin, isoniazid (INH), pyrazinamid and ethambutol for two months (the intensive phase), and rifampicin and INH for another four months (the continuation phase), according to WHO guidelines. In
the same period, patients with bacteriologically proven TB meningitis was included in Hasan Sadikin hospital in Bandung.

**Characterization of pulmonary tuberculosis patients**

Before start of TB-treatment, we recorded signs and symptoms, and history of possible previous TB-treatment or other diseases. Microscopic examination of acid-fast bacilli (AFB) in sputum slides was done by Ziehl-Neelsen staining, and graded according to the International Union Against Tuberculosis and Lung Disease (IUATLD) scale. Chest X-rays were read independently by two experienced radiologist who were blinded to the spoligotyping results and clinical information. Radiological lesions were classified as minimal lesions (mild), moderately advanced, and far advanced lesions and the presence or absence of pulmonary cavities was recorded (20). Blood examination included complete blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and fasting blood glucose. HIV-testing was performed after the study on recorded anonymous samples (Determine dipsticks, Abbott diagnostics, Hoofddorp, The Netherlands). Sputum microscopy and culture were repeated after the intensive phase (2 months), and at the end of treatment (6 months). We measured adherence by interview and pill-count. Previously we have compared several methods for measurement of adherence to treatment. The results showed the mean adherence in this setting, as measured with Medication Event Monitoring System (MEMS), to be 94% (21).

**M. tuberculosis culture, drug susceptibility testing and genotyping**

Culture for *Mycobacterium tuberculosis* was done on Ogawa 3% egg based media. Drug susceptibility testing was performed on cultured isolates using an absolute concentration method with supranational control. This method has shown accuracy between 96 and 100% in 10 rounds of WHO/IUATLD proficiency testing (22). Previous comparison of this method used in Indonesia has shown good agreement for INH and rifampicin, but less for streptomycin and ethambutol testing (B. Alisjahbana, submitted for publication). For 173 isolates sequencing of the hotspot region of the *rpoB* gene was performed, cross-checking the phenotypic drug susceptibility testing for rifampicin (23, 24).

From all patients with positive cultures, *M. tuberculosis* isolates were genotyped by spoligotyping. Spoligotyping of *M. tuberculosis* was done using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the manufacturer’s protocol and performed on DNA extracted from fresh colonies on Ogawa 3% media by boiling method (25). *M. tuberculosis* Beijing genotype was defined as a spoligopattern showing hybridization to at least three of the nine spacers 35 to 43 and absence of hybridization to spacers 1 to 34 (26). If no hybridization was found after repeated spoligotyping, the patient was excluded.
Spoligotyping was done in Hasan Sadikin Hospital, Bandung, Indonesia, but for 10% of isolates, and for all isolates not showing hybridization, spoligotyping results were confirmed in the Netherlands. Spoligotyping was repeated if *M. tuberculosis* cultures were still positive after two and / or six months of TB-treatment.

**Data analysis and statistics**

We compared the clinical and laboratory findings between TB patients infected with *M. tuberculosis* Beijing strains and those infected with non-Beijing strains. We used Pearson’s Chi-square to compare ratios, student T-test for normally distributed continuous variables, and Mann-Whitney non parametric for non-normally distributed continuous variables. Two-sided P-values < 0.05 were considered significant. Relative risks (95% confidence intervals) were calculated for bacterial response to treatment. Multiple regression was used to adjust for possible confounding. Data were analyzed using the Statistical Package for Social Sciences (SPSS Inc, Chicago, IL version 16.0).

**Results**

**Genotyping of patients with pulmonary and meningeal tuberculosis**

*M. tuberculosis* isolates were collected from 844 patients with a positive sputum smear. Twenty six were excluded from further analysis because repeated spoligotyping of isolates showed no hybridization (n=25) and one isolate was contaminated. For comparison, *M. tuberculosis* isolates from 148 patients with tuberculous meningitis were used. Spoligotyping was successful in 134 patients (90.5%) in this group. Among the total number of 952 patients with successful spoligotyping included for further analysis, no statistical correlation was found between Beijing genotype and disease localisation. *M. tuberculosis* Beijing genotype strains were found in 273 patients (33.4%) with pulmonary tuberculosis, and 38 (28.4%) of patients with tuberculous meningitis (NS).

**Presentation of patients with pulmonary tuberculosis according to genotype**

Pulmonary tuberculosis patients infected by *M. tuberculosis* Beijing (n=273) and non-Beijing genotypes (n=545) showed a similar sex distribution, age, duration and nature of symptoms (Table 1). Malnutrition, defined as BMI < 18.5 kg/m², was present in 59.7% of patients infected with Beijing genotype strains, and in 63.3% of patients infected with non-Beijing strains (NS). No association was found between genotype and radiological abnormalities. Mycobacterial load, as measured by sputum microscopy, was higher among patients infected with *M. tuberculosis* Beijing strains, although this was not statistically significant (P=0.06). Rates of drug resistance seemed higher among *M. tuberculosis* Beijing strains, but
this was not statistically significant for INH (P=0.31) nor rifampicin (P=0.59). No difference was found in inflammatory markers from laboratory examination (Table 1).

Table 1. Clinical characteristics of pulmonary tuberculosis according to M. tuberculosis genotype

<table>
<thead>
<tr>
<th></th>
<th>Beijing genotype (n=273)</th>
<th>Other genotypes (n=545)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>57.1</td>
<td>53.8</td>
</tr>
<tr>
<td>Age, median years (IQR)</td>
<td>30 (23-42)</td>
<td>30 (23-41)</td>
</tr>
<tr>
<td>History of TB contact</td>
<td>51.7</td>
<td>52.9</td>
</tr>
<tr>
<td>Previous treatment for TB</td>
<td>5.2</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Signs and symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration, median weeks (IQR)</td>
<td>8 (4-16)</td>
<td>8 (4-16)</td>
</tr>
<tr>
<td>Cough</td>
<td>98.9</td>
<td>98.9</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>39.6</td>
<td>44.1</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>71.8</td>
<td>67.3</td>
</tr>
<tr>
<td>Weight loss</td>
<td>81.7</td>
<td>82.4</td>
</tr>
<tr>
<td>Night-sweats</td>
<td>74.7</td>
<td>71.0</td>
</tr>
<tr>
<td>Body temperature ≥ 38°C</td>
<td>17.9</td>
<td>15.3</td>
</tr>
<tr>
<td>Median BMI (IQR)</td>
<td>17.8 (16.0-20.0)</td>
<td>17.8 (16.3-19.5)</td>
</tr>
<tr>
<td>BCG-scar present</td>
<td>49.1</td>
<td>45.1</td>
</tr>
<tr>
<td>Fever (^a)</td>
<td>72.5</td>
<td>79.2</td>
</tr>
<tr>
<td>Fever during treatment</td>
<td>21.8</td>
<td>14.8</td>
</tr>
<tr>
<td>Chest X-ray severity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced (^b)</td>
<td>53.7</td>
<td>56.4</td>
</tr>
<tr>
<td>Cavity present (^c)</td>
<td>65.0</td>
<td>64.0</td>
</tr>
<tr>
<td><strong>Sputum microscopy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive +</td>
<td>27.1</td>
<td>35.4</td>
</tr>
<tr>
<td>Positive ++</td>
<td>23.8</td>
<td>21.8</td>
</tr>
<tr>
<td>Positive +++</td>
<td>47.6</td>
<td>40.9</td>
</tr>
<tr>
<td><strong>Drug resistance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH resistant (^d)</td>
<td>10.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Rifampicin resistant (^e)</td>
<td>6.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Multidrug resistant (^f)</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Laboratory test results (median, IQR)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, mg/dL (^g)</td>
<td>12.0 (11.0-13.1)</td>
<td>12.0 (10.6-13.2)</td>
</tr>
<tr>
<td>Leucocyte count, 10(^9)/mL</td>
<td>11.0 (8.0-13.0)</td>
<td>11.0 (9.0-13.0)</td>
</tr>
<tr>
<td>Blood sedimentation rate, mm/hour (^h)</td>
<td>82 (62-105)</td>
<td>85 (60-107)</td>
</tr>
<tr>
<td>C-Reactive Protein, mg/dL (^i)</td>
<td>56 (30-94)</td>
<td>61 (29-86)</td>
</tr>
<tr>
<td><strong>Co-morbidity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Diabetes mellitus (^j)</td>
<td>16.6</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Note: Data are presented as %, unless indicated otherwise. BCG, Bacille Calmette-Guerin; BMI, body mass index; INH, isoniazid; int exclu, intermediate results were excluded, IQR, interquartile range; MDR, multidrug resistant.

\(^a\) P = 0.035

\(^d\) Data were available for 244 patients infected with Beijing strains and 500 patients infected with other genotype strains. Because of insufficient quality of radiographs, the presence of cavities could only be evaluated for 117 patients (48.0%) infected with Beijing strains and 261 patients (52.2%) infected with other genotype strains. Data are for 165 patients infected with Beijing, and 316 patients with other genotype strains, \(^b\)179 Resp. 347 pts, \(^c\)239 resp. 481pts, \(^d\)254 resp 503, \(^e\)239 resp. 466pts, \(^f\)105 resp 232, \(^g\)210 resp. 434pts, \(^h\)193 resp 394.
Reponse to treatment
During the intensive phase, mortality (0.4%), default (3.9%) and transfer (1.8%) were low and not different between groups. Adherence to treatment, as measured by patient’s reports and pill counts, was equally high in both groups (78.9% in Beijing group and 81.6% in non-Beijing group). The proportion of patients infected with *M. tuberculosis* Beijing strains developing a febrile response (>38.0°C) during treatment was slightly higher among patients infected with Beijing strains (21.8% vs 14.8%), as found earlier in Indonesia (27), although this difference was not statistically significant (P=0.11) Sputum microscopy after two months could be performed in 90.7% of patients who had started treatment, showing no difference between groups. Sputum culture after two months, performed in 71.1% of patients still in treatment, was more often positive in patients infected with *M. tuberculosis* Beijing strains, but this was not statistically significant (Table 2).

After six months, more treatment failures were found among patients initially infected with *M. tuberculosis* Beijing genotype strains. Culture after six months was performed in 450 patients, 55.0% of those who were still in treatment at this point (Table 2). Almost twice as many patients who initially were infected with a Beijing genotype strain had a positive sputum culture after six months (RR 1.95; 95% CI 1.25-3.02, P=0.003). This effect was not due to a higher rate of drug resistance among Beijing strains: patients initially infected with drug-sensitive Beijing genotype strains were also at higher risk of having a positive sputum culture after six months treatment (RR 1.74, 95% CI 1.08-2.80). Besides the Beijing genotype, other risk factors for treatment failure included rifampicin resistance (RR 4.11; 95% CI 2.03 – 6.83), and diabetes mellitus (RR 1.99; 95% CI 1.14 – 3.46), as previously reported (19). Multiple regression showed that the Beijing genotype was an independent risk factor for a positive sputum culture after tuberculosis treatment (P=0.03).

Spoligotyping of *M. tuberculosis* isolates from sputum collected during and after treatment confirmed the relation between the Beijing genotype and bacteriological response to treatment. Spoligotyping was successful among 65 patients who had positive sputum cultures after two months treatment, and 50 patients who had positive cultures at the end or shortly after tuberculosis treatment. At two months, the Beijing genotype was found in 40.6% of patients who had a positive sputum culture, and at six months, this was true for 50% of patients with positive sputum cultures, compared to 32.7% before start of treatment, P=0.03.
Table 2. Bacteriological response to treatment according to *M. tuberculosis* genotype.

<table>
<thead>
<tr>
<th>Bacteriology</th>
<th>Beijing (273 patients)</th>
<th>non-Beijing (545 patients)</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 2 months treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum microscopy performed (no. patients)</td>
<td>243</td>
<td>499</td>
<td>1.09 (0.82-1.43)</td>
</tr>
<tr>
<td>AFB-positive</td>
<td>16.0%</td>
<td>14.4%</td>
<td></td>
</tr>
<tr>
<td>Sputum culture performed (no. patients)</td>
<td>172</td>
<td>374</td>
<td></td>
</tr>
<tr>
<td>Culture positive for <em>M. tuberculosis</em>, no. patients (%)</td>
<td>22.7%</td>
<td>18.2%</td>
<td>1.24 (0.85-1.78)</td>
</tr>
<tr>
<td><strong>After 6 months treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum microscopy performed (no. patients)</td>
<td>185</td>
<td>374</td>
<td>1.39 (0.61-3.10)</td>
</tr>
<tr>
<td>AFB-positive</td>
<td>5.9%</td>
<td>4.3%</td>
<td></td>
</tr>
<tr>
<td>Sputum culture performed (no. patients)</td>
<td>150</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Culture positive for <em>M. tuberculosis</em>, no. patients (%)</td>
<td>24.0%</td>
<td>12.3%</td>
<td>1.95 (1.25-3.02) *</td>
</tr>
</tbody>
</table>

*p = 0.003

**Discussion**

We examined if the *M. tuberculosis* Beijing genotype was associated with an altered disease phenotype and treatment outcome among 954 tuberculosis patients in Indonesia. One third of patients were infected with *M. tuberculosis* Beijing genotype strains. We found no associations between Beijing genotype and disease localisation, clinical presentation or chest X-ray abnormalities. During prospective follow-up, patients infected with *M. tuberculosis* Beijing genotype strains experienced more treatment failure and more relapse, and this was not explained by differences in drug resistance.

So far, most studies examining possible explanations for the successful global spread of *M. tuberculosis* Beijing strains have focused on drug resistance, with several studies indeed showing higher drug resistance rates among Beijing genotype strains. However, Beijing strains may have additional properties which give them a selective advantage. In animal models Beijing strains have shown a higher degree of virulence, as reflected in more outgrowth, tissue damage and lower survival (8, 9). Few studies have examined the clinical phenotype of the *M. tuberculosis* Beijing genotype in tuberculosis patients, and ours is only the second prospective cohort study.

Our study provides no evidence to support our first hypothesis, that Beijing genotype strains would have a higher propensity for dissemination to the central nervous system. This is in line with research among adult patients in Vietnam (28) and children in South-Africa (29). One might also hypothesize that more virulent *M. tuberculosis* strains would more easily reach the central nervous system, but at least for the Beijing genotype this is not supported by our data and the studies cited above.
The second hypothesis we have examined was if *M. tuberculosis* Beijing genotype strains cause more severe pulmonary tuberculosis. Our data suggest that this is not the case. Signs and symptoms were rather similar. No difference was found in duration of symptoms (a shorter duration would suggest a higher virulence), cachexia or hemoptysis reflecting tissue damage, or any other symptom recorded. Our study is one of the largest and most detailed reported so far, and its findings are generally in line with previous studies, none of which have found dramatic differences in terms of signs and symptoms between patients infected with Beijing and other genotype strains (10, 12, 17). Similar to signs and symptoms, no association was found between genotype and radiological appearance, which is in line with some (13, 17) but not all previous reports (10, 12).

The third hypothesis we have examined is if Beijing strains are associated with a different response and outcome to tuberculosis treatment. Our data suggest that this is indeed the case. A higher proportion of patients infected with Beijing genotype strains developed fever during treatment, similar to what was found in a smaller study from Indonesia (27). The release of mycobacterial PAMPs (pathogen associated molecular patterns) during treatment may elicit a febrile response, and one might hypothesize that PAMPs from *M. tuberculosis* Beijing strains are more ‘immunogenic’ or released in higher quantities (30, 31).

In addition, patients infected with Beijing strains had an almost two-fold higher risk of having a positive *M. tuberculosis* culture after completing tuberculosis treatment. Among 224 Taiwanese patients, in the only other prospective cohort published so far, the Beijing genotype was also associated with treatment failure, albeit only among elderly patients (17). Earlier cross-sectional studies have shown relations between the Beijing genotype and tuberculosis treatment failure in Russia and Vietnam (10, 16, 32). Our study provides further support for a relation between Beijing genotype and bacteriological response to treatment as Beijing genotype strains made up a larger proportion among patient isolates after two months rather than before treatment, and again larger after six months versus two months treatment. We are the first to show that the higher risk of treatment failure among patients infected with Beijing strains was not due to differences in drug-resistance. This finding suggests that this genotype has certain characteristics *per se* which render them more resistant to tuberculosis treatment.

Our study has certain limitations. Data on drug resistance testing, chest X-ray examination and blood testing were incomplete, although this was equally a problem in both groups. Compared to other studies this study has several strong points. It is the second and largest comprehensive cohort study systematically and prospectively describing treatment outcome of patients infected with *M. tuberculosis* Beijing genotype strains. Very few patients were lost to follow-up,
and sputum culture, the most important endpoint was performed in a high proportion (71% of patients after two months, and 55% after six months). Patients without a culture had mostly recovered, rendering them unable to expectorate sputum. Adherence, which was well-monitored was high, excluding it as a confounder. Finally, other risk factors, including diabetes, HIV and drug-resistance were included in the analysis.

In conclusion, our understanding of the relationship between genotype and phenotype is still far from complete, but this study lends further support to the idea that differences in mycobacterial genotype indeed result in differences in disease phenotype, which may contribute to the predominance of mycobacterial genotypes like Beijing. Tuberculosis treatment was less effective against Beijing genotype strains, and this was not explained by drug-resistance. Further research should be conducted to explore the underlying mechanisms as well as the possible implications for tuberculosis treatment.

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References


Chapter 6

Death, recurrent tuberculosis and lung function impairment after tuberculosis treatment in Indonesia

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Submitted
Abstract

After tuberculosis (TB) treatment, patients remain at increased risk of death and recurrent TB. We examined long-term effects of pulmonary TB by comparing ex-TB patients with matched controls in Indonesia. Two-hundred ex-TB patients and 200 matched healthy controls from a previous case-control study were re-examined 1.5 to five years later. Measurements included survival, TB recurrence, chest-X-ray, pulmonary function, and sputum examination.

Data could be retrieved from 146 ex-TB patients (73%, 359 personyears), and 178 controls (89%, 388 personyears). All controls were alive, while three ex-TB patients had died after initial TB-treatment (p=0.055). Fourteen ex-TB patients developed recurrent TB, and three controls a first episode of TB (RR=5.2; 95% CI 1.5 – 17.7). Functional measurements showed more pulmonary restriction (41% vs 11%) and airway obstruction (10% versus 1%) in ex-TB patients compared to controls. TB recurrence was more frequent and lung function impairment more severe in patients initially infected with Mycobacterium tuberculosis Beijing genotype strains.

TB-patients remain at risk for recurrent TB and impaired pulmonary function. M. tuberculosis Beijing genotype strains seem more virulent. Follow-up of TB patients after treatment seems warranted and possible interventions to limit lung damage during TB-treatment should be examined.

Introduction

Tuberculosis (TB) is a major global health problem, killing about 1.7 million people every year (1). Treatment of tuberculosis is effective for the majority of patients. However, after successful treatment, TB may still impair health. First, patients die more frequently after treatment of TB compared to the general population. Reported mortality rates range from 1.5 – 10% in the first two years after end of treatment (2-6). Second, those who remain alive are at risk for TB recurrence, with a median recurrence rate 1780 per 10.000 personyears one year after treatment (7) reported rates ranging from 0.9% in pan-susceptible and mono-resistant TB patients, up to almost 100% in MDR-TB cases roughly two years following treatment. Besides drug resistance other factors have been related to death and recurrent TB including HIV infection, older age, diabetes, M. tuberculosis genotype, smoking and non-compliance (2, 3, 5, 7-13).

Third, as a result of destruction of lung tissue, TB can have long-lasting effect on general health. Shortly after TB treatment, pulmonary function is greatly impaired (14-16). One may postulate that patients will also suffer from more longstanding impairments.
We aimed to assess the long-term effects of pulmonary tuberculosis comparing ex-TB patients and matched healthy controls from a case-control study previously conducted in an ambulatory setting in Indonesia, which has the third highest TB-case-load worldwide (1). We measured survival, incidence of (recurrent) TB and pulmonary function.

**Methods**

**Subjects**

From a cohort of 481 TB-patients and 622 controls included in a case-control study conducted between 2001 and 2005 (17), 200 ex-TB patients and 200 matched healthy subjects were included. In the initial study, all patients had bacteriological proven pulmonary tuberculosis, while none of the controls had symptoms or chest X-ray findings suggesting TB. Inclusion, matching and data collection in the initial study have been described elsewhere (17). Patients were treated with a standard four-drug regimen according to national guidelines. Patients were classified as successfully treated if their initial treatment outcome was ‘cure’ or ‘treatment completed’ according to standard definitions (1), and unsuccessfully treated if their initial treatment outcome was ‘failure’ or ‘default’. Characteristics of subjects at time of enrolment in the previous case-control study are summarized in Table 1. For the current study all study subjects were invited to the clinic by letter. For subjects that did not come to the clinic, follow-up phone calls and maximum two home visits were done by a social worker during which structured interviews were conducted and subjects were asked to visit the clinic. Oral and written informed consent was obtained, dated and signed for all participating study subjects. Ethical clearance for the study was granted by the review committee of the Medical Faculty, Padjadjaran University.

**Measurements**

Data collection took place from April 2007 until August 2007. For subjects presenting at the clinic, height and weight were measured, signs and symptoms were recorded and TB status in the period since the initial study was assessed. Fasting Blood Glucose (FBG) was measured, and diabetes mellitus (DM) was diagnosed if the FBG concentration was >126 mg/dl. Chest X-ray examination was performed in all subjects visiting the clinic except pregnant women. In subjects with suspected tuberculosis (cough > 3 weeks, haemoptysis and/or abnormalities suggestive of TB on chest X-ray), sputum microscopy and *M. tuberculosis* culture was performed using solid media (3% Ogawa) (18). A long-term negative treatment outcome was defined as: death or new/recurrent TB following previous inclusion (controls) or TB-treatment (ex-TB patients). Lung function was assessed by spirometry using SpirobankG (19). Subjects were asked to stand and use a nose
clip during measurements. Subjects performed the following manoeuvres twice: calm inspiratory vital capacity (IVC), calm expiratory vital capacity (EVC) and forced vital capacity (FVC). Curves were followed real-time and measurements were repeated if necessary. The best result, within 15% of the range of total six manoeuvres was taken as vital Capacity (VC). The second outcome parameter was FEV1 /FVC ratio (best FEV1 and a FVC within 15% of the range of total six manoeuvres). Reference values of VC were obtained from an Indonesian survey among 1892 males and 1636 females (Pneumobile project, Boehringer, 1992). For those who had died, verbal autopsy (VA) was obtained from the closest contact at time of death. The verbal autopsy form was constructed using the WHO Standard Verbal autopsy (20) as guidance. Using verbal autopsy the cause of death was classified by a medical doctor as due to bacteriologically confirmed TB, probable TB (treated or hospitalized for suspected TB), possible TB (symptoms suggesting TB), other natural causes, or no natural cause.

Data analysis and statistics
Binominal outcome variables were tested by logistic regression, resulting in an Odds Ratio (OR) or p-value or cross-tabs resulting in Relative Risk (RR) or p-value by Pearson chi-square or Fisher’s exact test. Continuous outcome variables were tested by linear regression or by student’s T-test. Confidence intervals were calculated using single proportions or Poisson distribution for rare events. Differences in pulmonary function were determined using linear and logistic regression analysis. We included body mass index (BMI), smoking, age, gender, diabetes, and initial treatment outcome in the multivariate models.

Results
One hundred and forty-six of 200 ex-TB patients (73%) and 178 of 200 controls (89%) could be traced (Table 2). Ex-TB patients who were traced were similar in age, gender, initial treatment outcome, body mass index (BMI), diabetes prevalence and income at baseline to those who could not be traced and the same was true for controls (data not shown). Total follow-up time was 359 personyears for ex-TB patients and 387 for controls, ranging from three months to five years after the initial study. Two ex-TB patients were found to have died during the initial TB-treatment and were not included in the calculations. None of the traced subjects was HIV positive at the time of the initial study.

Three ex-TB patients (2.1%; 95% CI 0.4 - 6.0%) and none of the controls died during follow-up (p=0.055; Table 2). All deaths were due to TB, one after complete and two after incomplete TB-treatment. Based on treatment history and sputum examination, 14 ex-TB patients had developed recurrent TB, and three
controls a first episode of TB (RR=5.2; 95% CI 1.5 – 17.7). Nine ex-TB patients had a history of recurrent TB treatment, on average 1.6 years (95% CI 0.9 – 2.3) after their initial TB treatment, and two controls reported having had a first episode of TB (RR=5.8; 95% CI 1.3 – 26.5). Furthermore, of 233 study subjects who visited the clinic, six (5.5%) ex-TB patients and one (0.8%) control were found to have bacteriological positive TB, of whom one ex-TB patient already reported a TB episode after the initial study. Total negative long-term outcome was found in 17 ex-TB patients (5.7/100 personyears), and three controls (0.87/100 personyears), (RR=6.2; 95% CI 1.9 – 20.4). Patients that were successfully treated for TB remained significantly more at risk of recurrence (RR=3.9, 95% CI 1.1 – 13.8) and negative long-term outcome (RR 4.2; 95% CI 1.2 -15.0). Ex-TB patients initially infected with *M. tuberculosis* Beijing genotype strains had a higher risk of a negative outcome than ex-TB patients infected with other genotype strains (OR=8.6: 95% CI 1.4 – 53.7), this was also true when ex-TB patients who had defaulted or failed initial treatment were excluded (OR=12.2: 95% CI 1.0 – 145). Smoking had an effect on the long-term treatment outcome but this was not statistically significant for a history of ever smoking (OR=2.3: p=0.19), nor for pack-years (OR=1.1; p=0.052). Of 14 individuals with diabetes who came to the clinic, two (14%) had a negative long-term outcome compared to 12 (6.2%) of non-diabetic subjects (p=0.25). Two ex-TB patients (50%) with an initial episode of rifampicin-resistant TB had a negative long-term outcome compared to 13 (19%) with an initial episode with a pan-susceptible strain (p=0.15). None of the ex-TB patients with recurrent TB had a previous episode of isoniazid-resistant TB.

Spirometric examinations were done in 73 (37%) ex-TB patients and 84 (42%) controls. No differences in age, sex, initial treatment outcome, BMI, smoking, diabetes prevalence and income existed between ex-TB patients and controls who performed spirometry and those who did not. Adjusted to Indonesian reference values, Vital Capacity (VC) and FEV1/FVC (Forced Expiratory Volume in the first second/Forced Vital Capacity) were significantly lower for ex-TB patients than for controls (Table 2), also after exclusion of subjects with TB during follow-up. Among ex-TB patients, women had a lower VC than men (mean 78% vs. 85%, p=0.05), but this did not account for the significant difference in VC between ex-TB patients and controls. VC was lower among ex-TB patients with abnormal X-rays (74% vs. 91%; p<0.01). Pulmonary impairment did not change with increased smoking volume, and smoking did not account for the significant spirometric differences between ex-TB patients and controls (data not shown). Both VC and FEV1/FVC were significantly lower among 18 ex-TB patients initially infected with *M. tuberculosis* Beijing genotype strains compared to 33 ex-TB patients initially infected with strains belonging to other genotypes (VC: p<0.05; FEV1/FVC: p<0.05), and for VC this difference remained significant after exclusion of ex-TB patients with recurrent TB during follow up (p<0.05). More ex-TB patients than
controls had a VC below 80% of the predicted value (41% vs. 11%; OR=6.3; 95% CI 2.7 – 14.9), and a FEV₁/FVC below 70% (10% versus 1%; OR=8.7; 95% CI 1.0 – 72.4). When only successfully treated ex-TB patients were compared to controls, significance remained for both outcome measures. Almost sixty percent of ex-TB patients had an abnormal X-ray; those ex-patients had a significantly lower VC than ex-TB patients with a normal X-ray. Functional impairment seemed permanent: differences in VC and FEV₁/FVC were also found among subjects with more than four years of follow-up (data not shown).

Table 1. Baseline characteristics, collected in initial case-control study, of subjects that were invited in the follow-up study

<table>
<thead>
<tr>
<th></th>
<th>Ex-TB patients (n=200)</th>
<th>Controls (n=200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age - years (IQR)</td>
<td>26 (22-36)</td>
<td>26 (22-37)</td>
</tr>
<tr>
<td>Male sex</td>
<td>41.0</td>
<td>39.0</td>
</tr>
<tr>
<td>Median BMI - kg/m² (IQR)</td>
<td>18 (16-20)</td>
<td>21 (19-24)</td>
</tr>
<tr>
<td>Diabetes Mellitus #,**</td>
<td>10.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Smoking</td>
<td>41.9</td>
<td>50.3</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Initial TB-treatment successful</td>
<td>84.8</td>
<td>NA</td>
</tr>
<tr>
<td>Isoniazid resistance</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Rifampicin-resistance</td>
<td>6.6</td>
<td>NA</td>
</tr>
<tr>
<td><em>M. tuberculosis Beijing genotype</em></td>
<td>38.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are represented as % unless stated otherwise. # Status of diabetes mellitus was known for 160 ex-TB patients and 146 controls, HIV-status for 197 ex-TB patients and 64 controls, drug–resistance for 121 ex-TB patients, and spoligotyping results for 128 ex-TB patients. IQR = interquartile range; BMI = body mass index (kg/m²); NA= not applicable. P-values were based on student’s T-test (age, BMI), Fischer’s exact test (HIV) and chi-square (sex, smoking, diabetes).

* p<0.05; ** p<0.01.
Table 2. Characteristics of subjects that were traced during the follow-up study

<table>
<thead>
<tr>
<th></th>
<th>Ex-TB patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number retrieved (%)</td>
<td>146 (73%)</td>
<td>178 (89%)</td>
</tr>
<tr>
<td>Median follow-up - years</td>
<td>2.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Total follow-up - personyears</td>
<td>359</td>
<td>387</td>
</tr>
<tr>
<td>Death</td>
<td>2.1 (0.4 – 6.0)</td>
<td>0</td>
</tr>
<tr>
<td>Median BMI (IQR)*</td>
<td>21 (19-24)</td>
<td>22 (19-26)</td>
</tr>
<tr>
<td>Reported TB-treatment during follow up period **</td>
<td>7.4 (3.4 - 14.1)</td>
<td>1.3 (0.2 - 4.6)</td>
</tr>
<tr>
<td>Chronic cough</td>
<td>3.6 (1.0 – 9.3)</td>
<td>1.6 (0.2 – 5.8)</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>1.8 (0.2 – 6.6)</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal X-ray; possible TB **</td>
<td>57.7 (47.9-67.6)</td>
<td>4.5 (0.6-8.3)</td>
</tr>
</tbody>
</table>

Sputum examination

<table>
<thead>
<tr>
<th></th>
<th>n = 33</th>
<th>n = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB-positive (n)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Culture-positive (n)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>AFB or culture-positive (n)</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Lung function

<table>
<thead>
<tr>
<th></th>
<th>n = 73</th>
<th>n = 84</th>
</tr>
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<tbody>
<tr>
<td>mean VC - % predicted **</td>
<td>81.2</td>
<td>95.4</td>
</tr>
<tr>
<td>mean FEV1 / FVC ratio **</td>
<td>81.6</td>
<td>86.6</td>
</tr>
</tbody>
</table>

Table 2. Data are represented as %, (95% Confidence Interval based on Poisson distribution) unless stated otherwise. BMI was assessed in 109 ex-TB patients and 123 ex-controls. Reported TB (yes / no) could be established in 121 ex-TB patients and 157 controls, signs and symptoms were recorded in 110 ex-TB patients and 124 controls, and chest X-ray examinations were done in 97 ex-TB patients and 112 controls. BMI= body mass index (kg/m²); AFB = acid-fast bacilli; VC=vital capacity; FEV1= forced expiratory volume in one second; FVC= forced vital capacity. P-values were based on Fischer’s exact test (death, chronic cough, haemoptysis), linear regression analysis (BMI, lung function), and logistic regression analysis (abnormal X-ray, reported TB).

* p<0.05; ** p<0.01.

Discussion

Our study shows that Indonesian ex-TB patients more often die or develop (recurrent) TB and longstanding lung damage compared to healthy controls, even after successful treatment. Interestingly, one particular M. tuberculosis lineage, the Beijing genotype was associated with more frequent recurrence of TB and more severe lung damage than other genotypes.

Mortality during follow-up was higher among ex-TB patients than among healthy controls of similar background and (young) age, although this difference was not statistically significant. Mortality following TB-treatment in our study was 2.1%, which is lower than 6% mortality in a study in Vietnam (6), and 24% in Uzbekistan (2), while the follow-up in those studies was slightly shorter than ours (median 19 and 22 months respectively). Our findings for mortality may be an underestimate
as only 73% of ex-TB patients could be retrieved. Another possible explanation for the difference with previous studies is that our ex-TB patients, recruited in ambulatory care, were in a better medical condition and mostly (92%) newly diagnosed. Severe malnutrition and drug resistance were uncommon, HIV incidence was very low, and death during initial treatment was <1% (21).

Through anamnesis and active case-finding the risk of TB was six times higher in ex-TB patients than in healthy controls. To our knowledge, we are the first to compare risk of TB following treatment using matched healthy controls. Despite the fact that controls (in this endemic setting) were not screened nor treated for latent TB, they had a much lower risk of developing active TB than ex-TB patients who had received a full course of anti-TB drugs. This is an intriguing finding for which there are broadly three explanations. First, active TB may not have been fully eradicated during initial treatment, leading to relapse. Second, damage caused by an episode of active TB permanently impairs the first-line host defense of subjects, thereby making them more susceptible for recurrent TB due to reinfection. Finally, genetic and other factors affecting innate host defense may predispose subjects to progress to active TB following primary infection as well as following re-infection with *M. tuberculosis* after successful treatment. Immunogenetic and other studies are slowly unraveling why roughly 90% of individuals is able to contain *M. tuberculosis* infection, and 10% is not (22). The rate of reinfection after treatment of TB is higher than the rate of new TB in a high incidence setting (23), which might be explained by immunogenetic predisposition.

Interestingly ex-TB patients initially infected with *M. tuberculosis* strains of the Beijing family had more recurrent TB. The Beijing genotype is found in more than 30% of Indonesian TB-patients (23, 24) and is predominant in Asia (25). Its wide distribution suggests that the Beijing genotype family has a selective advantage. Beijing strains might acquire drug resistance more easily or might be more virulent, thereby hampering their eradication. This is consistent with our results and previous studies in Vietnam (9) and Singapore (26), which showed a higher relapse rate among patients infected with Beijing strains, even when corrections were made for drug resistance.

The third endpoint of the study was lung function. History of TB was strongly associated with impaired lung function, even years after successful treatment. Apparently, treatment of TB did not prevent permanent damage in this setting, although it cannot be excluded that lung damage was already present before the TB period. The prevalence and pattern of lung function impairment was similar to what was reported in other studies during and after treatment (14-16), although less severe. This impairment was equally severe among ex-TB patients regardless of when the initial episode of TB took place, suggesting that lung damage is
longstanding or even permanent, an important finding given the young age of ex-TB patients in this setting. The presence of functional impairment is in line with the fact that more than half of ex-TB patients had an abnormal chest X-ray, ex-TB patients with an abnormal X-ray having a significantly lower VC than ex-TB patients with a normal X-ray. Obstructive lung disease can be treated, but loss of lung tissue (restriction) cannot be repaired. Interestingly, also for lung function impairment *M. tuberculosis* Beijing genotype proved to be a risk factor, in line with animal studies (27). Beijing strains might elicit a different host response with more lung damage, but further studies in humans are needed to test this hypothesis.

Retrieval of information from ex-TB patients and to a lesser extent from controls proved difficult in this setting. Although their disease severity and treatment response at time of initial treatment were similar, it is still possible that TB recurrence or death were more common among those who could not be traced back. Only a minority of subjects (none out of three controls and 15 out of 45 ex-patients) who were asymptomatic but who had chest X-ray abnormalities presented for sputum examination, another reason why TB-recurrence may in fact have been higher. Prolonged and more intense follow-up of ex-TB patients, although difficult in settings like Indonesia, may help confirm our findings and lead to earlier detection and treatment of recurrent TB.

In summary, we have found that Indonesian ex-TB patients, even after successful treatment, have a higher risk of developing recurrent TB, and that a history of TB causes long-term impairment of lung function. Both endpoints are associated with infection by a particular *M. tuberculosis* genotype family. Further study is needed to assess optimal management of lung function impairment after an episode of TB.

**Acknowledgements**

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Tuberculosis caused by *Mycobacterium tuberculosis* Beijing genotype strains is associated with polymorphisms in *SLC11A1/NRAMP1* in Indonesian patients

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Abstract
Differences in host immune genes may predispose to tuberculosis caused by particular *Mycobacterium tuberculosis* genotypes. We have examined this hypothesis in Indonesia by spoligotyping *M. tuberculosis* isolates from 336 pulmonary tuberculosis patients, and typing the patients’ gene SLC11A1 (formerly called NRAMP1) which is involved in susceptibility to tuberculosis. The *M. tuberculosis* Beijing genotype, which made up 29.8% of all isolates, was strongly associated with two polymorphisms in SLC11A1; the D543N G allele (OR 2.15, P=0.005), and the 3’UTR ins/ins genotype (OR 2.5, P=0.0013). This supports the hypothesis of co-evolution of *M. tuberculosis* and the human immune system.

Introduction
Different evolutionary lineages of *M. tuberculosis* are strongly associated with specific geographical regions suggesting they have adapted to particular human populations. Indeed, particular *M. tuberculosis* lineages (also called: genotype families) have shown preferential spread in certain patient populations [1,2]. This may be due to differences in environmental exposure, but it might also be the result of co-evolution of host and pathogen. In this case, differences in host immune genes would confer increased susceptibility – or resistance – of certain human populations to particular *M. tuberculosis* genotype families. So far, only one study has tried to show a direct association between genetic characteristics of tuberculosis patients and their (own) mycobacterial isolates. In this study from Vietnam it was shown that a particular variation in toll-like receptor (TLR)2, a relevant receptor for *M. tuberculosis*, was more common among patients infected with strains belonging to the successful *M. tuberculosis* Beijing genotype than among patients infected with other genotype strains [3]. This study suggests that the outcome of exposure to *M. tuberculosis* depends on both human and bacterial genotype. We examined this hypothesis in Indonesia, which has the third highest global burden of tuberculosis. We have previously shown that 33% of tuberculosis patients in a large cohort in Indonesia were infected with strains belonging to the *M. tuberculosis* Beijing genotype [4], which globally is one of the most predominant families of *M. tuberculosis* [5]. In this same population we have now examined if infection with the Beijing genotype *M. tuberculosis* was linked to specific polymorphisms of SLC11A1, formerly called NRAMP1 (natural resistance-associated macrophage protein 1), a gene that was reported to be associated with susceptibility to tuberculosis, especially among Asian subjects [6].
Methods

From January 2001 through December 2006, consecutive patients over 16 years of age with microscopically proven pulmonary tuberculosis were included in two out-patient clinics and two hospitals in Jakarta and Bandung (West Java, Indonesia), as part of a case-control study examining host susceptibility to tuberculosis [7]. The diagnosis of tuberculosis was based on clinical presentation, chest X-ray examination, microscopic detection of acid-fast bacilli by Ziehl-Neelsen stained sputum smear, and culture of *M. tuberculosis* on 3% Ogawa medium. All patients were tested for HIV-infection, and HIV-seropositive patients (1.8%) were not included in further analysis. The study was approved by the ethical committee of the Faculty of Medicine, University of Indonesia, Jakarta and the Faculty of Medicine, Padjadjaran University, Hasan Sadikin Hospital, Bandung.

Spoligotyping was performed on *M. tuberculosis* sputum cultures from 769 tuberculosis patients. Mycobacterial DNA was extracted by bringing two loops of bacterial mass from a *M. tuberculosis* culture in saline and subsequent heating at 95°C for 5 min. Spoligotyping was performed using a commercial kit (Isogen Bioscience, Maarssen, The Netherlands). The presence or absence of 43 spacers in the DR region of isolates of *M. tuberculosis* was detected as described previously [4]. *M. tuberculosis* Beijing genotype was defined as a spoligopattern showing hybridization to at least three of the nine spacers 35 to 43 and absence of hybridization to spacers 1 to 34. Spoligotyping was done at the Hasan Sadikin Hospital, Bandung, Indonesia. For quality control, spoligotyping of 10% of the isolates, and all isolates lacking hybridization, was repeated at Gelre Hospital, Apeldoorn, the Netherlands.

Typing of *SCL11A1* polymorphism was performed in 342 patients of this cohort, but six HIV-infected patients were excluded from further analysis. Genomic DNA was isolated from EDTA blood. Two single nucleotide polymorphisms in the gene *NRAMP1*, D543N (1703G>A in exon 15 leading to an aspartate to asparagine substitution at codon 543, SNPId rs17235409) and INT4 (469 +14G>C in intron 4, SNPId rs3731865), as well as a TGTG insertion/deletion polymorphism in the 3’ untranslated region (1729+55ins/del4, SNPId rs17235416), denoted as 3’UTR were analysed as described before [8]. The Hardy–Weinberg equilibrium of each polymorphism was checked using the program HWE. The program CONTING was used to calculate χ² and associated values for a contingency table. All statistical analyses were two-sided and P values <0.05 were considered as statistically significant. Associations of *SCL11A1* polymorphisms with *M. tuberculosis* Beijing strain (as opposed to other or ‘non-Beijing’ strains) infection were expressed as odds ratios (95% confidence intervals).

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Results
Among 336 patients included, the median age was 30 years (range 16 - 75), 57.4% were male, and 5.1% had a history of previous tuberculosis treatment. One hundred (29.8%) were infected with *M. tuberculosis* Beijing genotype strains. The remaining 236 patients were infected with genotypes T1 (11.1%), Haarlem (9.3%), LAM (6.3%), EAI (4.2%), U/H3 (3.6%), remaining genotypes (4.7%), and with ‘orphan strains’ (31.0%), that had no shared-type with the international spoligo database SpolDB4. These results were representative for the total cohort of 740 pulmonary tuberculosis patients which was previously described [4]. Patients infected with *M. tuberculosis* Beijing strains and other (‘non-Beijing’) genotype strains were not significantly different in terms of age, gender or history of previous tuberculosis treatment. All 336 patients were HIV-seronegative.

The distribution of alleles and genotypes of three *SCL11A1* polymorphisms among patients infected with *M. tuberculosis* Beijing genotype and other genotype strains is shown in table 1. Each polymorphism was in Hardy-Weinberg equilibrium in the total group of patients as well as in 363 controls analyzed in a previous study [8]. The variant allele of INT4 was present in less than 5% of subjects, and therefore not further analyzed. As can be seen, significant associations were found between infection with *M. tuberculosis* Beijing genotype strains, and the two remaining *SLC11A1* polymorphisms. The G allele and the GG phenotype of the D543N polymorphism were significantly associated with infection by *M. tuberculosis* Beijing (*P*<0.01 and *P*=0.005 respectively). The association between *M. tuberculosis* Beijing genotype and the GG phenotype of D543N showed an OR of 2.15 (95% CI 1.25-3.70). Similarly, the ins allele and ins/ins genotype of the 3’UTR polymorphism were significantly associated with infection by *M. tuberculosis* Beijing genotype strains (*P*=0.01 and *P*=0.0013 respectively). The association between Beijing genotype strains and the ins/ins genotype of 3’UTR showed an OR of 2.40 (95% CI 1.19-4.83).
Table 1.  

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele or genotype</th>
<th>Frequency in pts infected with Beijing strains, n (%)</th>
<th>Frequency in pts infected with other genotype strains, n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D543N</td>
<td>G</td>
<td>172 (87.8)</td>
<td>360 (79.3)</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>24 (12.2)</td>
<td>94 (20.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>76 (77.6)</td>
<td>140 (61.7)</td>
<td>0.005a</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>20 (20.4)</td>
<td>80 (35.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2 (2.0)</td>
<td>7 (3.1)</td>
<td></td>
</tr>
<tr>
<td>INT4</td>
<td>C</td>
<td>193 (98.5)</td>
<td>457 (98.9)</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>3 (1.5)</td>
<td>5 (1.1)</td>
<td></td>
</tr>
<tr>
<td>3’UTR</td>
<td>ins</td>
<td>104 (88.1)</td>
<td>227 (77.7)</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>del</td>
<td>14 (11.9)</td>
<td>65 (22.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ins/ins</td>
<td>48 (78.0)</td>
<td>87 (59.6)</td>
<td>0.0013b</td>
</tr>
<tr>
<td></td>
<td>ins/del</td>
<td>12 (20.3)</td>
<td>53 (36.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>del/del</td>
<td>1 (1.7)</td>
<td>6 (4.1)</td>
<td></td>
</tr>
</tbody>
</table>

a GA and AA genotypes and b ins/del and del/del genotypes combined for analysis

Discussion

We report a strong association between host and bacterial genotype among tuberculosis patients in Indonesia. Patients carrying the most common genotypes of two different polymorphisms of SLC11A1 had a much higher chance of having tuberculosis caused by M. tuberculosis Beijing genotype strains than patients carrying other genotypes of these SLC11A1 polymorphisms. The human gene SLC11A1, formerly known as NRAMP1, is one of the most important genes reported to be associated with susceptibility to tuberculosis [6], but this is the first study to show a relation between SLC11A1/NRAMP1 polymorphisms and one of the most prevalent and most successful M. tuberculosis lineages, the Beijing genotype family.

The SLC11A1/NRAMP1 gene has been extensively examined for its association with tuberculosis.[6] It encodes a transmembrane protein which is exclusively expressed in macrophages and polymorphonuclear leukocytes. Upon phagocytosis, the SLC11A1 protein is rapidly recruited to the phagolysosomal membrane, where it mediates transport of iron (Fe^{2+}) and other cations. In addition, SLC11A1/NRAMP1 has pleiotropic effects on macrophage activation. Microbial pathogens, including M. tuberculosis, have homologues of SLC11A1. Iron is essential for biological functions, both for host immune defense and for
mycobacterial growth, and iron transporters of the human host and the mycobacterium compete intracellularly for iron [9]. SLC11A1 / NRAMP1 may also play a role in susceptibility to other intracellular pathogens including Salmonella.

We found that polymorphisms of SLC11A1 / NRAMP1 were associated with tuberculosis caused by strains belonging to one particular lineage of \textit{M. tuberculosis}, the Beijing genotype family. This genotype family is one of the most widespread evolutionary lineages of \textit{M. tuberculosis} [5]. All \textit{M. tuberculosis} lineages have expanded in the modern era, but the Beijing family has had by far the largest population increase [10]. This suggests that Beijing strains may have a selective advantage over other \textit{M. tuberculosis} strains. Indeed, Beijing strains have shown increased in vitro outgrowth in human monocytes and macrophages [11] and enhanced virulence and distinctive histopathology in animal models [12]. In human patients, Beijing genotype strains were associated with treatment failure and relapse [13], and with a higher chance to progress to active tuberculosis [14].

The association between the Beijing genotype and polymorphisms of SLC11A1 / NRAMP1 suggests these polymorphisms may increase susceptibility to infection or active tuberculosis caused by this particular genotype of \textit{M. tuberculosis}. Little is known about the functionality of the various polymorphisms of SLC11A1 / NRAMP1. However, it is very interesting that Beijing genotype strains were linked to the most common (and therefore evolutionarily the most successful) alleles of SLC11A1 / NRAMP1. These selected genotypes might lead to differences in transcription or translation of the SLC11A1-gene, or in different variants of the SLC11A1 protein which is involved in the bactericidal properties of macrophages. One might hypothesize that the Beijing genotype strains is better equipped to resist these selected variants of the SLC11A1 gene or protein than other \textit{M. tuberculosis} strains. In-vitro studies comparing outgrowth of different \textit{M. tuberculosis} strains in human macrophages with different SLC11A1 / NRAMP1 genotypes might help to further explore this hypothesis. Besides a direct effect from SLC11A1 / NRAMP1, the genetic association we have found might also indicate linkage to other immune genes in the vicinity of SLC11A1 / NRAMP1, such as the interleukin-8 receptors IL-8RA and IL-8RB. However, SLC11A1 / NRAMP1 seems a more likely candidate as so far no study has reported a significant role for the IL-8R in susceptibility to tuberculosis.

Globally, \textit{M. tuberculosis} shows strong geographical differences. We have previously typed \textit{M. tuberculosis} isolates from 897 patients originating from two different islands in Indonesia, Java and Timor [4]. Interestingly, a difference was found in the population structure of \textit{M. tuberculosis}. The Beijing genotype family was found in 33.0% of patients in Java, versus 14.3% of patients in Timor. Inversely, in Timor, the EAI and LAM genotype families were predominant, while
strains belonging to these genotypes were uncommon in Java. One could speculate about the explanation for these geographic differences. First, it may be due to a “founder effect”, with an increased chance of finding a particular \textit{M. tuberculosis} genotype family closer to where it originated. Secondly, the predominance of certain genotype families might be explained by an easier transmission or ‘escape’ from BCG-vaccination. However, our previous study did not support the latter hypothesis.\cite{4} Finally, as suggested by our current study, particular mycobacterial lineages may have adapted to specific properties of the immune system in particular human populations (‘genetic co-evolution’). Similar to geographical phylogenetic differences (‘phylogeography’) of \textit{M. tuberculosis}, the host immune genes also show geographical differences; we ourselves have shown a unique global distribution of two functional polymorphism of TLR4, one of the key pattern recognition receptors for a variety of pathogens, including mycobacteria \cite{15}. Similar to TLR4, SLC11A1 / NRAMP1 has a strong geographical variation \cite{6}, which might account for (part of) the geographical variation of \textit{M. tuberculosis}. Other host genes might be involved as well, and the Indonesian archipelago provides an excellent setting to further test the concept of human-mycobacterial ‘co-evolution’ as both \textit{M. tuberculosis} and the human host inhabiting the many islands most likely show substantial genetic variation.

\textbf{Conflict of interest}
None of the authors declares a conflict of interests

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References


Chapter 8

The successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains; a literature review on underlying mechanisms

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Submitted
Abstract
The wide geographic distribution of one clade of *Mycobacterium tuberculosis*, the Beijing genotype, and its genetic homogeneity suggests that strains belonging to this genotype may have a selective advantage over other *M. tuberculosis* strains. This hypothesis was addressed by reviewing molecular-epidemiological, experimental and clinical studies. Beijing strains represent 50% of strains in East-Asia and at least 13% of strains globally. Their emergence may be linked to BCG-vaccination, which seems less protective against Beijing strains, and to (multi-) drug resistance which is associated with the Beijing genotype in many areas. Different animal models have shown Beijing strains to be more virulent, causing more histopathology, higher outgrowth and increased mortality. At a molecular level, Beijing strains have specific properties in terms of protein and lipid structures and their interaction with the human immune system. Finally, the Beijing genotype has been linked to polymorphisms in a human immune gene, suggesting the possibility of human-mycobacterial co-evolution. All in all, the Beijing genotype family may represent an evolutionary response of *M. tuberculosis* making it more difficult to control through vaccination or antibiotic treatment. More research is needed to further unravel the mechanisms underlying the emergence of *M. tuberculosis* Beijing genotype strains, and examine the implications for tuberculosis control.

Introduction
The *Mycobacterium tuberculosis* Beijing genotype, which was first described in 1995 (1) is one of the most successful clades in the current worldwide tuberculosis (TB) epidemic (2-5). Initially, the Beijing genotype was recognized on basis of the highly conserved spoligo patterns and characteristic IS6110 restriction fragment length polymorphism (RFLP) patterns of *M. tuberculosis* isolates from the Beijing region and Mongolia (1). Since then, multiple studies have pointed out that the *M. tuberculosis* Beijing genotype strains have spread worldwide, are emerging in various areas, and often in association with (multidrug) resistance (2, 3, 5). As Beijing strains from different geographic areas show a remarkable degree of genetic conservation in comparison to other *M. tuberculosis* strains, one may hypothesize that this genetic lineage has selective advantages over other genotypes of *M. tuberculosis*, and have started spreading relatively recently. Previous reviews have described the spread and phylogeny of Beijing genotype strains (3, 5, 6), but this is the first comprehensive review bridging molecular-epidemiological, experimental and clinical studies related to these strains. If selection of Beijing strains and perhaps also other predominant genotypes represents the evolutionary response of *M. tuberculosis* to the major measures to fight tuberculosis, this may have serious implications for future plans on
tuberculosis control. Increased understanding of the mechanisms underlying the success of \textit{M. tuberculosis} Beijing strains may enable timely measures to prevent a further re-emergence of tuberculosis.

**Literature search strategy**

The literature reviewed was derived from an electronic search using Endnote database derived from Pubmed with the key words: (\textit{mycobacterium tuberculosis}[mh] OR tuberculosis[mh] OR \textit{mycobacterium tuberculosis}[ti] OR \textit{mtuberculosis}[ti] OR tuberculosis[ti]) AND (beijing[tiab] OR w-beijing[tiab] OR w-strain[tiab] OR w-variant[tiab]). Only articles published in English were selected. In total 292 articles were traced using these search terms. Additional studies were identified by searching the reference lists from existing articles.

**Phylogeny of \textit{M. tuberculosis} Beijing genotype strains**

Beijing genotype strains represent group 1 of the original grouping of Sreevatsan based on a limited number of single nucleotide polymorphisms (7), but are considered a grouping of the “modern” lineage of \textit{M. tuberculosis} according to the grouping of the \textit{M. tuberculosis} complex based on large sequence polymorphisms (8). The Beijing clade consists of at least two major groupings; the “typical and “atypical” Beijing strains (9-12). This distinction seems relevant, because these two lineages may have different properties (10), as will be discussed below. Beijing strains can also be subdivided in various evolutionary lineages through large sequence polymorphisms (LSPs), which can be used to divide this family into four monophyletic subgroups on basis of Regions of Difference (RD)105, RD181, RD150, and RD142 (13). The Beijing genotype of \textit{M. tuberculosis} has recently also been recognized as the major part of the ‘East-Asian lineage’ (14, 15).

**Recognition and molecular typing of Beijing strains**

In 1995, the Beijing genotype strains were defined on basis of their highly conserved spoligo patterns, revealing an almost invariable hybridization to the last nine spacers of the 43 spoligotyping spacers, and characteristic multi-banded \textit{IS6110} RFLP patterns (1). In terms of numbers, a minor sub-lineage of the Beijing genotype family, that caused multiple outbreaks among HIV positive patients in prisons and hospitals in New York in the 1990s was designated the “W” strain (16). However “W” strains only constitute a small branch on the phylogenetic tree of the Beijing genotype strains (11), and worldwide many of such phylogenetic branches exist. Therefore, we will refer to ‘Beijing strains’ as an indication of the worldwide Beijing genotype family as a whole.

Numerous techniques have been used to identify Beijing genotype strains. In 2004, based on extended study, \textit{M. tuberculosis} Beijing strains were defined as
strains hybridizing to at least three of the nine spacers 35 to 43 in the first generation spoligo spacers and absence of hybridization to spacers 1 to 34 (9). In addition, a standard set of 19 IS6110 RFLP patterns of Beijing strains was made available (www.tuberculosis.rivm.nl) to help identify Beijing strains in an IS6110 RFLP database. Rapid identification of Beijing strains is also possible in MIRU/VNTR typing by mycobacterial interspersed repetitive unit locus 26 (17). The second generation spoligotyping was able to distinguish different types of Beijing strains (18). It is not yet clear how this finding fits in with analysis of other phylogenetic markers. In the USA, a “W-Beijing poly-probe” assay was developed which targets include the genomic dnaA-dnaN and NTF region and the Direct Repeat locus, that are also used in spoligotyping. This method is highly sensitive and specific in the detection of Beijing strains among other M. tuberculosis strains (19). In 2005, a real time PCR which differentiates Beijing and non-Beijing strains was described (20).

Several methods with more discriminatory power have been proposed for epidemiological studies of Beijing strains. IS6110 RFLP typing initially had a higher discriminative power among Beijing strains than standard VNTR typing (21, 22), even with the use of additional loci selected in the latest 24-loci VNTR typing. However, more discriminatory power for Beijing strains has been established using 14 particular VNTR loci in addition to the exact tandem repeats (ETR) loci A to E (21). A 12-locus VNTR typing used in Japan is reported being superior in typing of Beijing strains to the 15-locus and 24-locus typing, and therefore seems the best tool for genotyping of M. tuberculosis isolates in areas where Beijing family strains are predominant (23).

In conclusion, Beijing strains from widespread geographic areas are genetically highly conserved. On the basis of various genetic markers we can clearly define M. tuberculosis Beijing strains and recognize its sublineages and individual strains.

Current spread of Beijing genotype strains worldwide
The fourth International Spoligotyping Database (SpolBD4), which classified 39,295 strains of M. tuberculosis from 141 countries into 62 clades/lineages, indicated that the Beijing and the Beijing-like strains represent about 50% of strains in East Asia, and 13% of the isolates globally (4). However, to date, spoligotyping has mostly been used in western countries, and data from many high prevalence settings, especially in South-East Asia and the former Soviet Union states, are missing. As a result, the true contribution of Beijing strains to the worldwide epidemic may exceed 30%. In the worldwide survey conducted within the framework of a European project, the proportion of TB attributable to the Beijing genotype varied significantly between areas: the prevalence in Asia was high, apart from the Indian subcontinent, increasing further east; low in parts of Africa, Latin America, and Western Europe; intermediate in the United States
and Cuba; low in Eastern Europe (other than the former Soviet Union); and low in the Middle East (2).

Several lines of evidence show that the Beijing genotype is emerging. First, a time trends analysis among non-immigrants from studies covering more than three years revealed a slight increase in the occurrence of Beijing genotype in all Western European countries (although only reaching statistical significance in The Netherlands) (2). Emergence of Beijing strains in this large study was defined as significant correlation between the Beijing genotype and low age. Young age is suggestive of active spread as was confirmed with DNA fingerprinting in the Netherlands, which showed that unique DNA fingerprints are associated with advanced age and DNA fingerprint clusters with young age (24). Whether this difference in the natural history of a tuberculosis in different age categories can be extrapolated to high prevalence countries remains to be proven.

As another approach to investigate possible dynamics in the population structure of *M. tuberculosis*, archived specimens have been used. In South Africa spoligotyping was applied to paraffin-embedded clinical material from consecutive time periods. In this area, Beijing strains were absent in histological samples from the period 1930–1965, rare in samples from 1966–1995, and increasingly common in samples from the period 1996–2005. The proportion of Beijing strains causing TB in children increased from 13% in 2000 to 33% in 2003 (25). Finally, a recent study determined the time of divergence, population diversity and spread of *M. tuberculosis* complex by MIRU-typing of a collection of 355 strains representing all well-defined primary branches of *M. tuberculosis* complex (26). Compared to other clades, the Beijing genotype displayed the largest population increase taken place in the last 180 years.

In conclusion, the Beijing genotype is the most predominant *M. tuberculosis* genotype in the world and there is clear evidence for its increase in certain areas.

**Factors contributing to the global emergence of *M. tuberculosis* Beijing strains**

The emergence of Beijing genotype may be due to natural selection, possibly skewed by the two major measures against TB in the last century: *Mycobacterium bovis* BCG vaccination and anti-TB treatment (27). BCG vaccination may be less protective against Beijing genotype strains than against other strains. Similarly, anti-TB treatment maybe less effective in eradicating Beijing strains than other strains. However, it has been suggested that the spread of Beijing strains already started long before the introduction of vaccination and antibiotic treatment (26), which would suggest that these strains have an intrinsic advantage over other *M. tuberculosis* genotypes in terms of transmission, progression from latent to active tuberculosis, acquisition of drug resistance or disease chronicity (28). Specific characteristics of this genotype may render it more virulent or better capable of
resisting or evading the human host immune system. We will discuss the different possibilities in more detail.

**Beijing genotype as an escape variant of BCG vaccination**

BCG vaccination may be a selective force favouring the spread of the Beijing genotype. In a BALB/c mouse (29) and a rabbit model (30) BCG vaccination was less protective against subsequent infection by Beijing strains than to infection with strains of other lineages. Another study, using C57BL/6 mice, was unable to confirm strain-specific resistance to BCG-vaccination (31). Newly developed candidate vaccines might be more protective; for instance the recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin ($\Delta$ureC hly+ rBC) vaccine induced a strong protection against infection by Beijing strains while parental BCG failed to do so (32). If BCG vaccination is indeed less protective against infection with Beijing strains, one would expect to find a higher proportion of Beijing strains among BCG vaccinated subjects compared to non-vaccinated individuals. This was indeed found in a study in Vietnam published in the year 2000, although the correlation was not statistically significant (33). Subsequent studies did not find any association (34-37). However when Beijing genotype isolates from The Netherlands and Vietnam were subdivided into a ‘typical’ and ‘atypical’ lineage, typical Beijing strains were indeed isolated more frequently from BCG-vaccinated than non-BCG-vaccinated persons (10). We may therefore conclude BCG vaccination is less protective against that particular lineage of the Beijing strains and may thereby promote selection of these strains. The underlying mechanism for this observation are still unclear.

**M. tuberculosis Beijing strain and drug resistance**

In the current era of tuberculosis-treatment, drug resistance may drive the spread of a particular *M. tuberculosis* genotype. Epidemiological studies have examined the association of drug resistance with Beijing strain (Table 1). A comprehensive review on this issue revealed four patterns: 1) endemic prevalence of Beijing strains, not associated with drug resistance 2) epidemic, associated with drug resistance 3) epidemic but drug sensitive and 4) very low level or absent (2). The difference between these patterns may be related to the variation in treatment regimens, compliance to treatment protocols, and different quality of drugs. Alternatively, it may be due to spread of different and not yet distinguished sub-lineages of the Beijing strains.

With regard to multidrug resistant (MDR) tuberculosis, it was recently shown that in countries with a high prevalence of Beijing strains, such as Azerbaijan, Ukraine, Uzbekistan, Estonia, Latvia, Lithuania, Mongolia and China, more than 5% of new tuberculosis cases involve MDR-TB (38). In Europe, in the period of 2003 to 2007, a total of 2.494 MDR-TB isolates from 24 European countries were subjected to
IS6110 RFLP typing to investigate transmission of MDR-TB across Europe (39)

About 39% of the examined cases were attributable to clusters and 84% of these likely transmissions were caused by Beijing strains. This is remarkable, because only 6-7% of the susceptible strains in Europe belong to the Beijing genotype. Strikingly, one very large cluster of 174 MDR-TB cases associated with the spread of one Beijing strain, was identified.

Logically, many researchers have examined possible associations between the Beijing genotype and the distribution of mutations in genes underlying resistance to anti-tuberculosis drugs (Table 2). Genotypic studies looking at katG315, the most important mutation encoding for resistance to INH, did indeed report higher mutation rates among Beijing strains (20, 40, 41). This is less clear for mutations in the rpoB gene, which account for >90% of drug resistance to rifampicin, as studies comparing Beijing and other genotype strains have examined the distribution (exact genetic location), rather than the overall frequency of rpoB-mutations. Some researchers found that the Beijing strains did not show significant differences in frequency of the most commonly encountered mutations in the rpoB gene compared to non-Beijing genotypes strains (42, 43). A significantly higher proportion of the rpoB S531L mutation in Beijing genotype strains was found in Germany and Russia. (20, 40), while in Korea researchers found the opposite (41). For mutations in the embB gene, associated with ethambutol (EMB) resistance, a study in Russia found that Beijing strains had a significantly higher rate of mutation in embB306 compared to non-Beijing strains, irrespective of their phenotypic susceptibility profiles. The Beijing genotype thus seems to acquire the most frequently arising (resistance) mutations more readily than do other genotypes, not only in embB306, but also in rpoB531 and katG315. On the other hand, EMB-susceptible, but MDR-TB, Beijing strains and non-Beijing strains showed similar rate of embB306 mutations. The author therefore hypothesized that an emergence of ‘silent’ embB306 mutations in EMB-susceptible strains may be predetermined not by the intrinsic genome structure (IS6110 RFLP profile) but rather by the previously acquired multidrug resistance (44). No studies have been published on associations of the Beijing genotype with other drug resistance genes like pncA, gyrA and rpsL/rrs, which are responsible for resistance against pyrazinamide, fluoroquinolone respectively streptomycin. Taking this information together, it is clear that more studies are needed to clarify this important issue.

From a mechanistic point of view, there are several explanations for a possible association between the Beijing strains and drug resistance. First, Beijing genotype strains may show a higher mutation frequency. It was previously found that Beijing strains have alterations in so-called ‘putative mutator genes’, resulting in altered DNA-repair and an increased mutation rate (45, 46). However, more recently it was found that the M. tuberculosis MutT2 (MtMutT2) gene plays a role in general slowdown of metabolism when mycobacteria are deprived of essential
nutrients (47). Later studies on 3R genes (DNA repair, recombination and replication genes) revealed that also strains of other M. tuberculosis genotypes have mutations in these genes and it is therefore not yet clear whether alterations in these genes play a significant role in the adaptation of Beijing strains (48). A second hypothesis is that specific characteristics of the cell-wall structure of Beijing strains lead to suboptimal intracellular concentrations of anti-tuberculosis drugs and acquisition of drug resistance (49-51). Both these hypotheses need further proof: in vitro, Beijing strains did not appear to acquire drug resistance more easily when exposed to anti-tuberculosis drugs (45, 52). Alternatively, increased virulence might lead to more persistent infections and treatment failures with prolonged exposure of Beijing strains to anti-tuberculosis drugs, and therefore more time to develop resistance.

In summary, many but not all epidemiological studies have shown associations between the Beijing genotype and drug resistance mutations. So far however, experimental studies and in-depth molecular studies have failed to reveal the underlying mechanisms for this association (as summarized in Table 1).

**Table 1.** Approaches to examine the relation of M. tuberculosis Beijing genotype with drug resistance

<table>
<thead>
<tr>
<th>No</th>
<th>Approach</th>
<th>Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Epidemiological studies of phenotypic drug resistance</td>
<td>associations with drug resistance in certain parts of the world, but not all</td>
<td>(2)</td>
</tr>
<tr>
<td>2.</td>
<td>Mutations in drug resistance genes</td>
<td>higher rates of mutations reported for rpoB codon S531L, katG, embB; different distributions of rpoB-mutations</td>
<td>(20,40,41)</td>
</tr>
<tr>
<td>3.</td>
<td>In vitro exposure to anti-TB drugs</td>
<td>no difference in acquisition of drug resistance</td>
<td>(52)</td>
</tr>
<tr>
<td>4.</td>
<td>Examination of underlying molecular mechanisms</td>
<td>more mutations in 'mutator genes' reported by some but not all</td>
<td>(45,46)</td>
</tr>
</tbody>
</table>

**Biochemical characteristics and immunogenecity of the Beijing genotype**

M. tuberculosis Beijing genotype strains may also be more virulent, as a result of intrinsic biochemical properties and their interaction with human host defense. In vitro studies have focused on protein expression, lipid structures and ‘immunogenecity’ of different M. tuberculosis genotypes (Table 2).

In a proteomic study, Beijing strains showed an increased expression of α-crystallin protein homolog or the 16-kDa protein, a M. tuberculosis virulence factor, and decreased expression of Hsp65, PstS1, and the 47 kDa protein compared to other clinical isolates and control strain H37Rv (53). Another proteomic study using human monocytic cell line U-937, showed that two proteins, Mb1363 (probable glycogen phosphorylase GlgP) and MT2656 (Haloalkane dehalogenase LinB), had a higher expression after phagocytosis of M.
Chapter 8

tuberculosis K-strain (a Beijing strain) compared to H37Rv, H37Ra and M. bovis BCG. It can be hypothesized that highly expressed GlgP protein in K-strains induces macrophage migration inhibitory factor (MIF) activation, which may be advantageous for the bacteria. Although the role of these proteins is not clear yet, they may have a critical function in the pathogenesis of tuberculosis (54).

Beijing strains also display differences in the cell-wall associated lipid structures. In vitro Beijing strains produce a biologically active lipid- a polyketide synthase-derived phenolic glycolipid (PGL), which was found to inhibit the release of pro-inflammatory mediators (55). In a more recent study, Beijing strains were found to accumulate large quantities of triacylglycerides in an in vitro aerobic culture. A particular glycolipid (PGL-tb), associated with lethal infection in animal models (55), was overexpressed in Beijing strains (50). Its expression coincided with upregulation of Rv3130c, a member of the DosR-controlled regulon of M. tuberculosis, which may confer an adaptive advantage for growth in microaerophilic or anaerobic environments during infection and thus may be related to the spread of this strain (50). However, PGL-tb was only synthesised by a subset of Beijing strains, so it is unlikely this is the only virulence factor.

Another hypothesis is that Beijing genotype strains induce a different immune response, or undermine an effective host response. This has been the focus of in vitro research and animal studies (Table 2). Infection of human monocytic THP-1 cells with Beijing genotype strains induced a lower production of several cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-12p40 compared to other strains (56). On the other hand, macrophages infected with Beijing isolates expressed higher levels of mRNA for iNOS, IL-1β, TNF-α, IL-12 cytokines and lower levels of IL-10 compared to cells infected with other genotype (57). This expression pattern has been associated with infection control, but during in vivo infection with Beijing strains progression to chronic phase was absent (57). Macrophages stimulated with lipid fractions of Beijing strains had a higher production of TNF-α and IL-10, but down regulation of TLR2, TLR4 and MHC class II expression (58). In contrast, lipids from a M. tuberculosis canetti strain induced lower amounts of TNF-α and IL-10, and upregulation of TLR2 and TLR4, without modifying MHC class II expression (58).

The highly virulent Beijing (‘K’) strain induced significantly higher levels of necrotic cell death rather than apoptosis in THP-1 cells than did H37Rv bacteria. These results suggest that Beijing strains keep cellular apoptosis as a host defense mechanism to a minimum and induce necrosis in macrophages (56).

**Beijing genotype strains in animal models**
Animal models indicate that Beijing genotype strains are more virulent and more effectively resist or evade the host immune response. BALB/c mice intratracheally
injected with Beijing strains showed more histopathology, bacterial outgrowth and higher and earlier mortality compared to animals infected with H37Rv and other genotype strains (29). This coincided with early TNF-α and inducible isoform of nitric oxide synthetase (iNOS) expression (29).

Similarly, when BALB/c mice were challenged with 19 different *M. tuberculosis* strains of 11 major genotype families, Beijing strains induced more severe histopathology and higher mortality (59). In a rabbit model, central nervous system infection with HN878 or W4 resulted in higher bacillary loads in the cerebrospinal fluid and brain, increased dissemination of bacilli to other organs, persistent levels of TNF-α, higher leukocytosis, and more-severe clinical manifestations compared the *M. tuberculosis* clinical isolate CDC1551 (a highly immunogenic strain) (51). Also in B6D2/F1 mice these same Beijing strains HN878 and W4 strains induced higher mortality compared to the highly transmissible strain CDC1551, coinciding with higher levels of type I IFNs, lower levels of TNF-α and IL-12 and reduced T cell activation (60). Intrathecal injection of BALB/c mice by a Beijing strain induced a twofold higher percentage of apoptotic activated macrophages than mice infected by H37Rv, and earlier progressive pneumonia which contained numerous macrophages, with vacuolated cytoplasm. Vacuolated macrophages might induce apoptosis of Th1 cells which favors disease progression, and is related to the virulence of the mycobacterial strains (61).

There are also indications in animal studies that the immunological response after infection by Beijing strains is less adequate than after infection by other strains (29, 30, 59). This could affect the required synergistic effect of the immunological response of a patient and drug treatment to cure a patient from tuberculosis, and create a situation of prolonged time to sputum conversion which in turn increases the risk of development of drug resistance.

Put together, there is clear evidence that Beijing strains show altered expression of proteins, glycolipids and triglycerides, which may contribute to increased virulence, or to evasion or suppression of protective host defense mechanisms. However, no conclusive evidence has been put forward to explain the overall success of Beijing strains in circumventing the BCG-induced immunity or enabling the development of resistance.
### Table 2. Biochemical characteristics, immunogenicity and virulence of *M. tuberculosis* Beijing genotype strains.

<table>
<thead>
<tr>
<th>Experimental Approach</th>
<th>Control Strain used</th>
<th>Findings associated with Beijing genotype strains</th>
<th>Interpretation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteomics</td>
<td>F 23 strain, H37Rv</td>
<td>↑ of α-crystallin ↓ of Hsp65, PstS1, 47 kDa protein</td>
<td>Increased virulence</td>
<td>(53)</td>
</tr>
<tr>
<td>Lipid expression</td>
<td>CDC1551, NHR5</td>
<td>↑ polyketide synthase-derived phenolic glycolipid (PGL)</td>
<td>Evasion of host immune response</td>
<td>(55)</td>
</tr>
<tr>
<td>Lipid expression; gene expression</td>
<td>H37Rv</td>
<td>↑ tricylglycerides (TAG) ↑ DosR regulon gene</td>
<td>Increased capability for latency and transmission, ↑ survival within host tissues.</td>
<td>(50)</td>
</tr>
<tr>
<td>Proteomics</td>
<td>H37Rv, H37Ra, Mt. bovis BCG</td>
<td>↑ Mb1363 (probable glycogen phosphorylase GipP), ↑ MT2656 (Haloalkane dehalogenase LinB)</td>
<td>Not yet determined</td>
<td>(54)</td>
</tr>
<tr>
<td><strong>Immunogenicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infection of murine macrophages</td>
<td>Canetti, H37 Rv</td>
<td>↑ mRNA for iNOS, IL-1β, TNF-α, IL-12 ↓ IL-10</td>
<td>Pro-inflammatory cytokine response</td>
<td>(57)</td>
</tr>
<tr>
<td>Lipid fraction vs human monocyte-derived macrophages</td>
<td>H37Rv, Canetti</td>
<td>↑ TNF-α and IL-10, ↓ TLR2, TLR4 and MHC class II expression</td>
<td>Pro-inflammatory cytokine response</td>
<td>(58)</td>
</tr>
<tr>
<td>Infection of human mononuclear THP-1 cells</td>
<td>H37Rv</td>
<td>↓ TNF-α, IL-6 and IL-12p40 ↑ necrotic cell death</td>
<td>Less apoptosis and more necrosis of infected macrophages</td>
<td>(56)</td>
</tr>
<tr>
<td><strong>Virulence in animal models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrathacal injection BALB/c mouse</td>
<td>H37Rv</td>
<td>↑ extensive pneumonia, early but ephemeral TNF-α, inducible (iNOS) expression, earlier mortality, ↑ bacillary load</td>
<td>increased virulence</td>
<td>(29)</td>
</tr>
<tr>
<td>Intrathacal injection BALB/c mouse</td>
<td>H37Rv , Canetti</td>
<td>severe pathological response, ↑ mortality, ↑ bacillary load</td>
<td>increased virulence</td>
<td>(59)</td>
</tr>
<tr>
<td>Intracisternal injection in rabbits</td>
<td>CDC1551</td>
<td>↑ bacillary load, ↑ dissemination of bacilli to other organs, persistent levels of TNF-α, ↑ leukocytosis, and more-severe clinical manifestations</td>
<td>increased virulence</td>
<td>(30)</td>
</tr>
<tr>
<td>Aerosol B6D2/F1 mice</td>
<td>CDC1551</td>
<td>↑ type I IFNs, ↓ TNF-α, IL-12, ↓ T cell activation, ↓ survival</td>
<td>increased virulence</td>
<td>(60)</td>
</tr>
<tr>
<td>Intratracheal injection BALB/c mouse</td>
<td>H37Rv</td>
<td>↑The peak progressive increase of apoptotic Th1 lymphocytes</td>
<td>increased virulence</td>
<td>(61)</td>
</tr>
</tbody>
</table>

↑ = higher/increased/large/overexpressed/more, ↓ = decreased/lower/downregulated

Beijing lineage family included: HN878, Beijing strain 210,W10,W4, K-strain

TBM = tuberculous meningitis
Clinical phenotype of Beijing strains: patient studies

If Beijing strains are more virulent one would expect a higher proportion of infected patients to develop active tuberculosis. This hypothesis is supported by a study in the Gambia. In a cohort of tuberculosis patients and household contacts, transmission rates between patients exposed to *M. tuberculosis* and *M. africanum* were similar, but rates of progression to disease were significantly higher in contacts exposed to a Beijing family strain (28). Differences between Beijing genotype and other *M. tuberculosis* complex genotypes were less clear, and the overall number of investigated cases was relatively small. Still, this is a promising hypothesis which needs further exploration.

Taking the relative success of Beijing strains into consideration one might also expect that patients infected with Beijing genotype strains present with a different or more severe clinical phenotype. Several studies have investigated this hypothesis. In a small study in Indonesia, patients infected with Beijing genotype strains more often developed fever unrelated to disease severity during the first stage of treatment (62), but this was not confirmed in studies from Singapore and Russia, which actually reported less fever and and night sweats in patients infected with Beijing strains (17, 57).

Studies comparing chest X-ray abnormalities of patients infected with Beijing and other genotype strains have also met with conflicting results. In Russia and Singapore associations were found between the extent and severity of radiological abnormalities (35, 37), while in The Netherlands, such associations were not found (34). However, it could be that the correlation between Beijing bacteria and resistance is much stronger in Former Soviet Union states than in The Netherlands, resulting in more persistent infections and, hence, more lung damage. A high bacterial load in sputum increases the risk of transmission but so far, no study has reported associations between bacterial load in the lung/sputum of patients infected with different genotype strains.

If Beijing strains are more virulent, this might also be reflected in a higher proportion of patients with disseminated disease. In Arkansas patients with extrapulmonary TB were found to have three times more likely to be infected with Beijing strains after correcting for potential confounders, however, in children there was no evidence of such an association in Cape Town South Africa (63). In Vietnam, HIV-infected patients with meningeal tuberculosis caused by Beijing strains had a shorter duration of illness prior to presentation, and a lower cerebrospinal fluid leukocyte count than patients with meningitis caused by other *M. tuberculosis* genotype strains (64). In conclusion, in some but not all studies, patients infected with *M. tuberculosis* Beijing strain showed clinically more severe and more rapid disease progression than patients infected by non Beijing strain.
Co-evolution of the Beijing genotype and humans

There must be an explanation for geographic differences in the population structure of *M. tuberculosis*. First, this may be due to a “founder effect”, with a higher chance of finding a particular genotype family closer to where it originated. The population structure may also be related to environmental factors or differences in TB control. Second, particular *M. tuberculosis* lineages may have adapted to specific properties of the immune system in particular human populations (‘genetic co-evolution’). Similar to geographical phylogenetic differences (‘phylogeography’) of *M. tuberculosis*, the host immune genes also show geographical differences. For instance, functional polymorphism in Toll-like receptor (TLR)4, one of the primary ‘pattern recognition receptors’ (PRR) for *M. tuberculosis* (65), show a unique global distribution (66). Several lines of research support the notion that the geographic variation of *M. tuberculosis* and the human host genotype are related. Particular *M. tuberculosis* lineages have shown preferential spread in particular patient populations (14, 67). However, people of the same background or immigration group tend to live together in a foreign country thereby spreading their ‘native strains’ to other people of the same ethnicity. On the other hand, preferential spread of a particular genotype in a specific ethnic group may also be the result of co-evolution of host and pathogen. However, so far only one study has tried to show a direct association between genetic characteristics of tuberculosis patients and the genotype of *M. tuberculosis* isolates. In this study in Vietnam it was shown that a particular mutation in TLR2, another key PRR for *M. tuberculosis* (65), was more common in patients infected with *M. tuberculosis* Beijing genotype strains than among patients infected with other genotype strains, suggesting that the outcome of exposure to *M. tuberculosis* can depend on both the human and bacterial genotype (68). These findings provide further support for the hypothesis that evolutionary adaptation of particular *M. tuberculosis* lineages to certain human populations contributes to the marked geographical variation *M. tuberculosis* genotypes, and that host gene polymorphisms, especially related to immune recognition of *M. tuberculosis*, confer increased susceptibility of certain populations to the Beijing genotype. Clearly, this concept of ‘genetic co-evolution’ needs further investigation.

Conclusion

Worldwide, there is an emergence of a genetically conserved genotype of *M. tuberculosis*; the Beijing strains, often in association with (multi-)drug resistance. This global change in the population structure of *M. tuberculosis* is most likely driven by man-made factors (antibiotic treatment and vaccination) and perhaps
also linked with intrinsic mycobacterial characteristics, as outlined in this review and summarized in Table 3. The Beijing genotype family has been studied most extensively so far. However, other predominant genotypes like the Haarlem and African genotypes may undergo similar changes. An evolutionary response of *M. tuberculosis* towards a new population of bacteria that are more difficult to treat and have a higher ability to circumvent vaccination will hamper our efforts to control tuberculosis. Therefore, there is an urgent need to better understand the mechanisms underlying the emergence of *M. tuberculosis* Beijing genotype strains, and examine the implications for tuberculosis control.

**Table 3.** Mechanisms contributing to the emergence of *M. tuberculosis* Beijing genotype strains

<table>
<thead>
<tr>
<th>No</th>
<th>Mechanism</th>
<th>Evidence</th>
<th>Implication</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>'escape' from BCG-vaccination</td>
<td>BCG vaccination less protective against subsequent Beijing strains infection in animal models</td>
<td>more latent infection</td>
<td>(29,30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Typical Beijing strains more frequently from BCG-vaccinated patients</td>
<td>more active TB</td>
<td>(10)</td>
</tr>
<tr>
<td>2.</td>
<td>adaptation (‘co-evolution’) to human immune genes</td>
<td>Association of TB caused by Beijing genotype with TLR-2 polymorphism in Vietnam</td>
<td>more active TB</td>
<td>(68)</td>
</tr>
<tr>
<td>3.</td>
<td>more progression from latent infection to active TB</td>
<td>More active TB among case-contacts of patients infected with Beijing genotype in The Gambia</td>
<td>more active TB</td>
<td>(28)</td>
</tr>
<tr>
<td>4.</td>
<td>more drug resistance</td>
<td>association of Beijing genotype with MDR-TB and drug resistance mutations in <em>rpoB</em>, <em>katG</em>, <em>embB</em> in cross-sectional studies</td>
<td>slower or absent response to TB-treatment</td>
<td>(20,40,41,44)</td>
</tr>
<tr>
<td>5.</td>
<td>higher virulence</td>
<td>Higher outgrowth and lower survival in animal studies</td>
<td>more progression and chronicity of TB (with or without antibiotics); higher bacterial load</td>
<td>(59,60,61)</td>
</tr>
<tr>
<td>6.</td>
<td>higher immunogenicity</td>
<td>Stronger pro-inflammatory cytokine response; more necrosis of infected macrophages</td>
<td>more cavitary disease with higher sputum bacterial load</td>
<td>(29,30,59)</td>
</tr>
</tbody>
</table>

**References**


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Chapter 9

Summary and General Discussion
Tuberculosis remains a major global health problem with an estimated 9.3 million new cases (139 per 100,000 people), including 1.37 million (15%) cases among HIV-positive people. In that same year, there were an additional 1.16 million relapse or retreatment TB cases. Multi-drug resistant (MDR) TB, which is defined as TB resistant to at least isoniazide (INH) and rifampicin, occurred in more than half a million people worldwide (1). These data clearly show that TB is far from being eradicated, despite the availability of BCG vaccination and effective anti-TB treatment. *Mycobacterium tuberculosis* has adapted to TB-treatment (developing drug resistance), to vaccination, or to the immune system of host, and this adaptation has contributed to its evolutionary success.

Since the development of molecular methods, it is clear that *M. tuberculosis* is not a single entity. To distinguish *M. tuberculosis* in more detail, several genotyping methods have been introduced. The most widely used techniques are restriction fragment length polymorphism (RFLP), spoligotyping and mycobacterial interspersed repetitive-unit–variable-number tandem repeat (MIRU-VNTR) typing (2-4). These methods allow us to detect certain *M. tuberculosis* genotypes, follow transmission between individual patients, study ‘outbreaks’ of tuberculosis, and distinguish relapse from re-infection in patients with recurrent tuberculosis. In addition, these methods have helped us to study the genetic relations (‘phylogenetics’) or dynamics of the population structure of *M. tuberculosis* complex. By MIRU typing, two major lineages have been defined, called clade 1 and 2. A further geographic sub-structuring within clade 1 revealed distinct branches for the African (Uganda, Cameroon and S), Asian (Beijing and CAS), Latin American-Mediterranean and African-European populations (X, Ghana and Haarlem). Clade 2 consists of both animal and human pathogenic isolates. All *M. tuberculosis* complex populations from human sources have shown markedly constant expansion rates in the last 180 years. But the Beijing genotype family which is one of the most successful lineages at present, has shown by far the largest increase in population size (5). The Beijing clade comprises at least 2 major subgroups, which share the characteristic spoligotype pattern (6-8): typical and atypical Beijing strains. Typical (“modern”) (8, 9) Beijing strains, showed highly similar, multicopy IS6110 restriction fragment length polymorphism (RFLP) patterns (10), while atypical (“ancestral”) (8, 9) Beijing strains more closely resemble the common ancestor of the Beijing clade (6-8).

Using spoligotyping for molecular genotyping, we found that the *M. tuberculosis* Beijing genotype is the predominant clade in Indonesia. The success of Beijing strains may be related to their ability to withstand tuberculosis treatment, or evade the host immune response. This is the focus of this thesis.
Summary of research findings

*M. tuberculosis* Beijing strains are highly prevalent in Indonesia, accounting for one third of population structure of *M. tuberculosis* complex. This is the first study of its kind in Indonesia which has a third highest rank of TB after India and China. I will now summarize the main findings of each chapter.

Before implementation, as this is the first molecular technique in a clinical laboratory in Bandung, Indonesia, I optimized spoligotyping for different sample sources by examining its reproducibility. In Chapter 2 I found that spoligotyping described by Kamerbeek et al (11) when it was used for non cultured *M. tuberculosis*, showed weak hybridization signals in some of the spacers. Therefore, we optimized our PCR mixture for direct spoligotyping of *M. tuberculosis* complex DNA in clinical samples by using a different concentration MgCl2, Tris-HCl, and PCR-primers (12). The use of this adjusted protocol yielded complete spoligotyping patterns for *M. tuberculosis* complex in clinical samples as compared with cultured bacteria.

The population structure of *M. tuberculosis* in Indonesia, and the prevalence of the Beijing genotype is the basis for this thesis. In Chapter 3 the distribution of various *M. tuberculosis* genotypes among almost 900 patients from two different islands was determined. A high degree of genetic diversity was observed among *M. tuberculosis* strains, and a significant difference was found in the geographical distribution of genotype families. The predominant Beijing genotype family was isolated from 33% of patients from West Java versus 14.3% of patients from Timor, with two other genotype families (East African-Indian and Latin American / Mediterranean) being more prevalent in Timor.

Increased rates of drug resistance might be one possible explanation for the predominance of *M. tuberculosis* Beijing genotype strains. In Chapter 4 I examined the association between *M. tuberculosis* Beijing genotype and drug resistance gene mutations among isolates from over 300 Indonesian tuberculosis patients. Sequencing of the rpo8-hotspot region and PCR for mutations in katG codon 315 and embB codon 306 was performed on phenotypically drug-resistant and –sensitive patient isolates. More drug resistance gene mutation were found in *M. tuberculosis* Beijing strains compared to non-Beijing strains, both for rpo8 (33.3% versus 18.7%), katG315 (18.5% vs. 12.8%), and embB306 (16.5% vs. 6.1%). This was not due to an overrepresentation of Beijing strains among patients with a history of previous TB-treatment: among newly treated patients, drug resistance gene mutations were also strongly associated with the Beijing genotype. Animal studies have shown that *M. tuberculosis* Beijing genotype are more virulent compared to other strains, but data from clinical studies are scarce. In a
prospective cohort study in Chapter 5, we examined if the Beijing genotype is associated with severity of tuberculosis and treatment failure in Indonesia. No clear association was found between Beijing genotype and disease presentation or chest X-ray abnormalities, but approximately twice as many patients who were initially infected with a Beijing genotype strain had a positive sputum culture after six months therapy. This effect was not due to a higher rate of drug resistance among Beijing strains. Multiple regression showed that the Beijing genotype was an independent risk factor for a tuberculosis treatment failure.

After treatment of tuberculosis, patients remain at increased risk of death, recurrent TB, and permanent lung damage. The long-term effects of pulmonary TB caused by Beijing strains and non-Beijing strains was compared in Chapter 6. One hundred and forty six ex-TB patients and 178 matched healthy controls from a previous case-control study were re-examined 1.5 to five years later. Ex-TB patients initially infected with *M. tuberculosis* Beijing genotype strains had a higher risk of recurrent TB than ex-TB patients infected with other genotype strains. Lung function impairment was much more pronounced among 18 ex-TB patients initially infected with *M. tuberculosis* Beijing genotype strains compared to 33 ex-TB patients initially infected with strains belonging to other genotypes. Differences in host immune genes may confer increased susceptibility to particular *M. tuberculosis* genotypes, and this might be another contributing factor to the evolutionary success of Beijing strains. In Chapter 7 we examined this hypothesis by typing the human gene *SLC11A1*, formerly called *NRAMP1* (natural resistance-associated macrophage protein 1), which has been linked with susceptibility to tuberculosis in many settings, in 331 pulmonary tuberculosis patients. *M. tuberculosis* Beijing genotype, which was one third of all isolates, was strongly associated with two polymorphisms in *SLC11A1*; the D543N G allele and the 3'UTR ins/ins genotype. Indeed, certain genotypes of two different polymorphisms of *SLC11A1* were strongly associated with infection with *M. tuberculosis* Beijing genotype strains.

The wide geographic distribution of *M. tuberculosis* Beijing genotype strains, and its genetic homogeneity suggests that strains belonging to this genotype may have a selective advantage over other *M. tuberculosis* strains, as is supported by the studies presented in chapters 3-7. In Chapter 8 I reviewed all molecular-epidemiological, experimental and clinical studies related to the Beijing genotype that have been published so far. From the scientific literature, it appears that the evolutionary success of Beijing genotype strains may be linked to BCG-vaccination, which seems less protective against Beijing strains, and to (multi-) drug resistance which is associated with the Beijing genotype in many areas. In addition, different animal models have shown Beijing strains to be more virulent, causing more
histopathology, higher outgrowth and increased mortality. At a molecular level, Beijing strains have specific properties in terms of protein and lipid structures and their interaction with the human immune system. Besides our finding that the Beijing genotype was linked with polymorphisms in \(SLCA1/NRAMP1\), Beijing strains have also been linked to polymorphisms in the gene encoding for TLR2, an important pattern recognition receptor for mycobacteria, suggesting the possibility of human-mycobacterial co-evolution. In conclusion, the Beijing genotype family may represent an evolutionary response of \(M. \text{tuberculosis}\) making it more difficult to control through vaccination or antibiotic treatment.

**General Discussion**

Based on the separate studies as summarized above, we can make two important conclusions. First, the population structure of \(M. \text{tuberculosis}\) in Indonesia shows much heterogeneity, and geographical differences, but there is a strong predominance of strains belonging to a single clade, the Beijing genotype. This clearly suggests that this clade of \(M. \text{tuberculosis}\) has selective advantages over other clades. Second, several different factors may account for the predominance of Beijing genotype strains, including a higher virulence (especially a higher persistence during treatment), more drug resistance, and adaptation to the human innate immune system.

I will now discuss these findings in more detail focussing on four aspects: epidemiology, molecular characteristics, clinical phenotype, and relation with the host immune system.

The **first major finding** of this thesis was the high prevalence of \(M. \text{tuberculosis}\) Beijing genotype, which is remarkable given the strong heterogeneity of \(M. \text{tuberculosis}\) genotypes in Indonesia. This high genetic diversity of \(M. \text{tuberculosis}\) is in contrast with studies from other higher prevalence areas, including e.g. Vietnam, where only two major genotype families were found: Beijing (35-54%) and EAI (20%) (13-16). The \(M. \text{tuberculosis}\) Beijing genotype family was the most prevalent genotype family in Indonesia (33%), and the proportion of Beijing strains was stable during the study period from 2001 to 2006, and similar to the prevalence recorded in a previous, smaller study (32.4%) (17). Thus, the Beijing genotype seems to be an endemic strain circulating in Indonesia.

Interestingly, the Beijing genotype was much less frequent in Timor, an island in the eastern part of Indonesia, than in Java. One can hypothesize about the underlying cause for this difference. First, it may be due to a ‘founder’ effect with a higher chance of finding of particular genotype family closer to where it originated. As a second possibility, particular \(M. \text{tuberculosis}\) lineages may have adapted to specific properties of the immune system in particular human
population (genetic co-evolution). The results in chapter 7 support this hypothesis (18). Several studies have reported that the population structure of human pathogens is geographically structured, and this phenomenon has been linked to ancient human migration (5, 19, 20). The fact that there are different ethnic population in Java and Timor, suggest that there is an association of ethnic-specific host genetic factors with TB susceptibility. As the highly prevalent genotype in Timor were EAI (East African Indian) lineage (33.3%) and LAM (Latin-American-Mediterranean) lineage (20.0%) are also common in Far East Asia and Africa respectively (21), one can speculate that maybe these genotypes were associated with human migration from those areas (21). In contrast to what has been reported in others studies (13, 14, 16), no association was found between the Beijing genotype and age, previous treatment, phenotypic drug resistance and BCG vaccination.

Regarding BCG vaccination, no correlation between \textit{M. tuberculosis} Beijing and BCG vaccination, similar to other studies (22-25). Recently a study with strains from three different countries did find an association between the ‘typical’ (also called: ‘modern’) sublineage of Beijing strains and BCG-vaccination, suggesting that this sublineage can evade the immune protection induced by BCG-vaccination (26). In our studies in Indonesia, we did not further differentiate the Beijing genotype “typical” (“modern”) Beijing strains, and “atypical” (“ancestral”), Beijing strains (8, 9). As mentioned that only typical \textit{M. tuberculosis} Beijing strain was circumvent vaccine induced immunity (26). One can postulate that \textit{M. tuberculosis} Beijing strains circulating in Indonesia belong to the “atypical” or “ancestral” Beijing clade.

A \textbf{second major finding} in this thesis is the higher prevalence of mutations in drug resistance genes in \textit{M. tuberculosis} Beijing genotype strains compared to non-Beijing strains. More mutations were found in \textit{rpoB}, \textit{katG315} and \textit{embB306}, three genes which are responsible for resistance to rifampicin, isoniazide and ethambutol respectively. A first explanation for our finding is that Beijing genotype strains have a higher mutation frequency. It was previously found that Beijing strains have alterations in so-called ‘putative mutator genes’, resulting in altered DNA-repair and an increased mutation rate (27, 28). However, more recently it was found that the \textit{M. tuberculosis mutT2} (MtMutT2) gene plays a role in general slowdown of metabolism when mycobacteria are deprived of essential nutrients (29). Secondly, the high prevalence of drug resistance gene mutations in Beijing strain might be due to a founder effect, with a particular strain with certain genetic characteristics circulating in this area. However, the strong genetic variability of \textit{M. tuberculosis} isolates in this area (24), collected from different sites over a 6-year period of time, argues against bias of our findings by one particular (resistant) cluster. We found a difference between Beijing and non-
Beijing strains in the distribution of specific mutations in \textit{rpoB} (30). The most common mutation, Ser531Leu, which seems to have very little effect on fitness (31, 32), had a higher prevalence among non-Beijing strains, while other mutations (including those in codon 526), which seem to have a higher fitness ‘cost’ were more common among Beijing strains. Similar to a study among South African children, we found a stronger association of Beijing and \textit{rpoB}-mutations among newly diagnosed patients than among previously treated patients, suggesting easier transmission (more ‘fitness’) of resistant Beijing strains (33). In addition, we also found a strong correlation between mutations in \textit{embB306} and mutations in \textit{rpoB} and \textit{katG315}. This finding is supported by previous studies which suggest that mutations in \textit{embB306} may not only account for ethambutol resistance but also correlate with multiple drug resistance (34-37). Irrespective of the underlying mechanism, our study confirms that mutations in \textit{embB306} are highly predictive of multidrug resistance.

The higher rate of mutations in \textit{rpoB}, \textit{katG315} and \textit{embB306} among \textit{M. tuberculosis} Beijing strains provides further evidence that drug resistance may contribute to the global predominance of Beijing strains. The strong association of Beijing and \textit{rpoB}-mutations among new cases of TB reflects considerable transmission of resistant Beijing strains in Indonesia.

\textbf{The third major finding} in this thesis is that patients infected with \textit{M. tuberculosis} Beijing strains are at increased risk for treatment failure and long term damage. One may postulate about the underlying cause for this finding. The difference in our study was not due to difference in adherence or drug resistance. In fact, our study was the first to show that the Beijing genotype was also associated with positive sputum cultures after treatment completion among patients without drug resistance. This finding suggests that this genotype has certain characteristics per se which render them more difficult to eradicate with tuberculosis treatment. Possibly, specific characteristics of the cell-wall structure of \textit{M. tuberculosis} Beijing genotype strains contribute to their ability to withstand the host immune defense of tuberculosis treatment. For instance, the different cell wall may lead to suboptimal intracellular concentrations of anti-tuberculosis drugs and acquisition of drug resistance (38-41). These hypotheses need further proof: \textit{in vitro}, Beijing strains did not appear to acquire drug resistance more easily when exposed to anti-tuberculosis drugs (27, 42). Alternatively, increased virulence might lead to more persistent infections and treatment failures with prolonged exposure of Beijing strains to anti-tuberculosis drugs, and therefore more time to develop resistance. Earlier cross-sectional studies have shown relations between the Beijing genotype and tuberculosis treatment failure in Russia (23) and Vietnam (16), but those two studies were done in specific populations, in prison and in elderly patients respectively.
Similar to a higher rate of treatment failure, patients infected with *M. tuberculosis* Beijing strains also had more recurrent TB and more (long term) lung damage. One can postulate why this is the case. First, active TB may not have been fully eradicated during initial treatment, leading to relapse. Second, damage caused by an episode of active TB may have permanently impaired the first-line host defense of subjects, thereby making them more susceptible for recurrent TB due to re-infection. Finally, genetic and other factors affecting innate host defense may predispose subjects to progress to active TB following primary infection as well as following re-infection with *M. tuberculosis* Beijing strains.

As a **fourth major finding**, we found that polymorphisms of *SLC11A1 / NRAMP1* among tuberculosis patients were associated with infection by *M. tuberculosis* Beijing strains. *SLC11A1 / NRAMP1* is the most widely examined candidate gene for susceptibility to tuberculosis (43). In this thesis, it was shown that patients with a particular genotype of *SLC11A1*, which has often been linked with susceptibility to tuberculosis, had a much higher chance to be infected with Beijing strains. Adaptation of *M. tuberculosis* (‘co-evolution’) probably underlies this finding (18, 44). It may also explain why the prevalence of the Beijing genotype shows strong geographic differences (obviously the genotype of the human host is also very different across the Indonesian archipelago). So far, only one other study has similarly shown an association between *M. tuberculosis* genotype and host genotype (44).

**The success of Beijing strains – a conceptual framework**

From separate studies and discussion above we can generate a conceptual framework of factors underlying the success of *M. tuberculosis* Beijing genotype strains. In our study we did not find any association of Beijing genotype with BCG vaccination, but we still can not exclude that correlation. BCG vaccination may be less protective against typical Beijing genotype strains than against other strains. This will lead to more latent infection and more progression from latent infection to active TB (45-47). Similarly, anti-TB treatment maybe less effective in eradicating Beijing strains than other strains. Arguing against these two points, however, it has been suggested that the spread of Beijing strains already started long before the introduction of vaccination and antibiotic treatment. This would suggest that Beijing strains have an intrinsic advantage over other *M. tuberculosis* genotypes in terms of transmission, progression from latent to active tuberculosis, acquisition of drug resistance or disease chronicity (45). Specific characteristics of this genotype may render it more virulent as its associated with treatment failure and relapse (16, 23), and proven to cause more lung damage animal model or better capable of resisting or evading the human host immune system (39, 46-50). A conceptual framework of factors underlying the success of Beijing genotype is shown in **Figure 1**.
Chapter 9

Figure 1. A conceptual framework of factors underlying mechanism the success of *Mycobacterium tuberculosis* Beijing genotype.

**Overreaching issues**

Besides the specific questions addressed in this thesis, there are two overreaching or cross-cutting issues. First, the research findings have certain implications for public health and TB-control. Second, the theoretical framework and practical experience which are the basis for this thesis can help decide about the most effective way to implement or improve molecular typing of *M. tuberculosis* and mycobacteriology in general in Indonesia. I will now focus on these two overreaching issues: public health and laboratory aspects.

**Public health aspects**

In terms of public health, several issues warrant further discussion. This thesis focussed on the differences between *M. tuberculosis* Beijing and non-Beijing strains. But even without looking at these differences, important conclusions can be drawn. This thesis brings up several issues related to tuberculosis control in Indonesia. First, the Beijing strain which is predominant in Indonesia, is related with the higher transmission and treatment failure. As such it is an additional risk factor for failure, next to HIV co-infection, diabetes, low nutritional state and so on. In our setting, drop out is low and adherence is high (51). Second, the treatment failure
rate is high compared with data from countries using one of two standardized initial regimens, where failure rates average 5.0% (in countries where prevalence of initial MDR exceeds 3%), or 1.6% (in countries where initial multidrug resistance prevalence is less than 3%) (52). Besides treatment failure, recurrent TB also seems common: in our study of long-term effects of pulmonary TB, 9.6% of 146 ex-TB patients developed recurrent TB.

The issue of high treatment failure and high transmission rate has not been fully considered by the National TB program because the current DOTS program is considered quite sucsessful with >70% coverage and a decrease in the prevalence of TB. As the Beijing strains may be most threatening to the TB control program, to combat the emergence of this strain, certain measures should be explored, like the need for higher dose or prolonged treatment. Besides, strain identification should be considered at population level, or even maybe at the level of individual patients.

Third, significant rates of drug resistance were found. Although this is partly due to selection bias, this is still a matter of grave concern. So far, Indonesia has not been included in an international survey on TB drug resistance. Similarly, no national survey, and no single survey of sufficient size or quality has been published. In fact, so far only two laboratories have met international quality control. Both these laboratories only use the Canetti proportional method, which is 50 years old. Hardly any research and development is being done in drug susceptibility testing in Indonesia. Only this year, the national TB program has supported efforts to evaluate a commercially available genotypic method as a screening assay for drug resistant TB. Several years ago with help from the Dutch TB reference laboratory (RIVM) a 25-well plates method with transparent Middlebrook’s medium was introduced (53). Accuracy of this method was better, but it proved difficult to establish sustainable production of plates, and contamination during production and inoculation was a significant problem. As an alternative, we are now evaluating cheap in-house methods of genotypic drug susceptibility testing, and ‘MODS’ (Microscopic Observation Drug Susceptibility) assay for TB drug resistance, which is a very cost-effective method which has performed very well in other less-developed settings (54, 55).

Lack of infrastructure, human resources and funding are some of the obstacles towards high-quality drug susceptibility testing for TB in Indonesia. There is an urgent need for a national reference laboratory which is responsible for standardized identification and TB drug susceptibility testing, education on TB and biosafety, as well as external quality assurance. Good surveillance of drug resistance does not only require good laboratory practice, but also good epidemiology and good quality patient data.
As shown in this thesis, both issues discussed above, drug resistance and treatment failure, are associated with the Beijing genotype. This is an important finding, but the implications for patient management and TB control remain to be determined.

**Laboratory technique aspects**

Application of molecular techniques in clinical service laboratories will help rapid detection of *M. tuberculosis* complex and might play a role in improving routine susceptibility testing. In this study we found that microscopy was not sufficient for TB detection as would have missed at least 17% of failure cases if only microscopy had been used. While culture is time consuming, a rapid TB molecular based detection may be a useful method in this setting. Molecular techniques have the advantage of being quick and accurate. Although not cheap, they might have a role in management of difficult cases.

Molecular based drug susceptibility testing (DST) might have an even greater impact to TB control. Although techniques will be difficult to implement to all service laboratories in Indonesia, it could be done in provincial public health laboratories or provincial reference hospitals which are often equipped with molecular detection equipment. However, a supranational laboratory should be first established as a body responsible for quality assurance of molecular detection.

While the quality of routine methods should be strengthened in primary care settings like the Puskesmas, some form of ongoing surveillance looking at the quality of this primary diagnosis and presence of drug resistance should be done in Provincial referral laboratories. The referral lab should be closely linked to care of complicated TB patients, e.g. those who default or have multidrug drug resistant TB.

**Remaining issues**

Several questions are still unanswered; 1) what are the underlying mechanism of virulence associated with Beijing strains? 2) what is the population structure of *Mycobacterium tuberculosis* complex in other Indonesian islands? 3) what is the clinical phenotype of TB caused by Beijing strains? 4) what are the implications of all of this for TB control in Indonesia? These issues certainly need further study.

**Academic collaboration**

The studies in this thesis could be done through a fruitfull collaboration between Universitas Padjadjaran/Dr. Hasan Sadikin Hospital and University Medical Center Nijmegen, The Netherlands. Beside official university collaboration, this network was extended to other institutions either in The Netherlands, also in Indonesia.
The benefit of this collaboration is beneficial for patient care, education and research, through knowledge transfer, supervision, training, and sharing experience in tuberculosis diagnosis and care. This collaboration has helped to strengthen laboratory capacity in tuberculosis diagnosis using both conventional and molecular methods. We were able to improve our microscopic and culture method and evaluate ‘MIC 25 micro well plate’ for TB drug susceptibility testing. We also successfully established a molecular typing method, spoligotyping, which has been done on more than thousands isolates. Now, we are able to assist National Institute of Health (Badan Penelitian dan Pengembangan Kesehatan) Ministry of Health of Indonesia as a consultant and reference laboratory on National TB Molecular Epidemiology project, also contribute to evaluation phase of the Hain “GenoType® MTBDR plus” test for rapid detection of MDR-TB. Additional ongoing research under this collaboration are on molecular detection of \textit{M. tuberculosis}, real time PCR for MDR-TB, MODS etc. aim for patients service. Hopefully, in the future this collaboration will sustainable and extend, covering more staff of Medical Faculty Universitas Padjadjaran / Dr. Hasan Sadikin Hospital with mutual benefit and the ultimate goal to control TB.

Concluding remarks
This thesis helped to establish the clinical laboratory infrastructure and skills to address the molecular methods for detection, identification, drug resistance and epidemiology of \textit{M. tuberculosis} complex. This contribute to increase understanding of the underlying mechanism of the emergence of \textit{M. tuberculosis} Beijing strains. These comprehensive studies were only possible through good collaboration of many researchers and institutions in Indonesia and in The Netherlands. There is still much we should explore in order to more effectively fight TB. I therefore hope that this thesis can be another step in the understanding of TB, and in the development of new tools and strategies for TB control.

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Ringkasan dan Pembahasan Umum
Tuberkulosis (TB) masih merupakan masalah kesehatan utama yang mendunia dengan perkiraan 9.3 juta kasus baru (139 per 100 000 penduduk) termasuk 1.37 juta (15%) kasus diantara penderita positif HIV. Dalam tahun yang sama terdapat tambahan 1.16 juta kasus TB kambuh. *Mycobacterium tuberculosis* resisten ganda (*Multi Drug Resistant*) yang didefinisikan sebagai *M. tuberculosis* yang resisten terhadap paling sedikit isoniazid (INH) dan rifampisin, ditemukan pada lebih dari setengah juta penduduk di seluruh dunia (1). Data tersebut dengan jelas memperlihatkan bahwa TB masih jauh dari eradicasi walaupun ada vaksinasi BCG dan pengobatan anti TB yang efektif. *Mycobacterium tuberculosis* telah beradaptasi terhadap pengobatan TB (munculnya galur yang resisten obat), terhadap vaksinasi atau terhadap sistem imun pejamu, dan adaptasi ini berperan kepada keberhasilan perkembangan evolusinya.

Sejak perkembangan metode molekuler, jelas bahwa *M. tuberculosis* bukanlah mikroorganisme tunggal. Untuk membedakan *M. tuberculosis* secara lebih rinci, berbagai cara *genotyping* telah diperkenalkan. Teknik yang paling luas dipergunakan adalah *Restriction fragment length polymorphism* (RFLP), spoligotyping dan *Mycobacterial interspersed repetitive-unit–variable-number tandem repeat* (MIRU-VNTR) *typing* (2-4). Berbagai metode ini dapat mendeteksi genotip *M. tuberculosis* tertentu, mengikuti transmisi antar pasien, menyelidiki sumber kejadian luar biasa (*outbreaks*) TB, dan membentuk kasus yang kambuh dibandingkan dengan yang infeksi berulang. Sebagai tambahan, metode metabol ini telah membantu untuk meneliti kekerabatan genetik (*phylogenetics*) atau dinamika struktur populasi *M. tuberculosis* kompleks. Dengan MIRU typing, dua keturunan utama telah terdefinisi, disebut *clade* 1 dan 2. Pembagian secara geografis selanjutnya, *clade* 1 menghasilkan cabang-cabang yang berbeda untuk Africa (Uganda, Cameroon dan S), Asia (Beijing dan CAS), Latin America- Mediterranea dan populasi Africa-Eropa (X, Ghana and Haarlem). *Clade* 2 terdiri atas isolat patogen baik untuk manusia maupun hewan. Seluruh populasi *M. tuberculosis* kompleks dari sumber manusia menunjukkan kecepatan ekspansi yang konstan pada 180 tahun terakhir ini. Tetapi genotip Beijing yang merupakan salah satu dari keturunan yang paling sukses pada saat ini menunjukkan penambahan populasi yang paling besar (5). Beijing *clade* terdiri dari paling sedikit 2 grup utama yang mempunyai karakteristik pola spoligotype yang sama (6-8) yaitu galur Beijing *typical* dan *atypical*. Galur Beijing *typical* ("modern") (8, 9), memperlihatkan kesamaan yang tinggi pada pola *multi copy* IS6110 *restriction fragment length polymorphism* (RFLP) (10), sedangkan galur Beijing *atypical* ("ancestral") (8, 9) lebih cenderung sama dengan nenek moyang Beijing *clade* (6-8).

Dengan spoligotyping, kami menemukan bahwa genotip *M. tuberculosis* Beijing merupakan *clade* yang paling banyak di Indonesia. Keberhasilan galur Beijing diduga berhubungan dengan kemampuannya untuk bertahan dari pengobatan TB,
atau menghindar dari respons imun pejamu. Hal inilah yang menjadi fokus dari tesis ini.

Ringkasan temuan penelitian

Populasi *M. tuberculosis* galur Beijing banyak ditemukan di Indonesia, merupakan sepertiga dari struktur populasi *M. tuberculosis* kompleks. Ini merupakan penelitian pertama kali di Indonesia, negara dengan peringkat TB ke tiga tertinggi setelah India dan China. Berikut ini adalah ringkasan temuan utama dari setiap bab.

Teknik spoligotyping merupakan teknik molekuler pertama di laboratorium klinik di Bandung, Indonesia, oleh karena itu, pertama kali, kami melakukan optimasi teknik ini untuk berbagai sumber sampel yang berbeda dengan menguji reproduksibilitasnya. Dalam bab 2 saya menemukan bahwa spoligotyping yang telah dijelaskan oleh Kamerbeek dkk (11) ketika digunakan untuk *M. tuberculosis* yang bukan berasal dari hasil kultur, memperlihatkan sinyal hibridisasi yang lemah di beberapa spacer. Oleh karena itu, kami melakukan optimasi campuran PCR untuk spoligotyping DNA *M. tuberculosis* kompleks langsung dari spesimen klinik dengan menggunakan konsentrasi MgCl2, Tris-HCl, dan PCR-primers yang berbeda (12). Penggunaan protokol yang disesuaikan ini berhasil menunjukkan pola spoligotyping *M. tuberculosis* kompleks dengan lengkap pada sampel klinik sebanding dengan sampel yang berasal dari kultur.

Struktur populasi *M. tuberculosis* di Indonesia, dan prevalensi genotip Beijing adalah dasar dari tesis ini. Dalam bab 3 diteliti distribusi dari berbagai genotip *M. tuberculosis* di antara hampir 900-an pasien dari dua pulau yang berbeda. Keragaman genetik yang sangat tinggi ditemukan diantara galur *M. tuberculosis*, dan ditemukan perbedaan yang bermakna pada distribusi geografisnya. Genotip Beijing ditemukan sebanyak 33% dari pasien yang berasal dari Jawa barat berlawanan dengan sejumlah 14.3% dari pasien yang berasal dari Timor, dengan dua genotip famili (East African-Indian dan Latin American / Mediterranean) lebih banyak ditemukan di Timor.

Meningkatnya resistensi terhadap obat merupakan salah satu kemungkinan yang dapat menerangkan mengenai predominansi genotip *M. tuberculosis* galur Beijing. Dalam bab 4 saya menguji hubungan antara genotip *M. tuberculosis* Beijing dengan mutasi pada gen yang bertanggung jawab terhadap resistensi obat diantaranya isolat yang berasal dari 300-an pasien TB. Sekuensing regio hotspot *rpoB* dan PCR untuk mutasi pada gen *katG* kodon 315 dan gen *embB* kodon 306 dilakukan pada isolat yang secara fenotipik resisten maupun sensitif. Mutasi pada gen resisten lebih banyak ditemukan pada *M. tuberculosis* galur Beijing dibandingkan dengan galur non-Beijing, untuk *rpoB* (33.3% versus 18.7%),
katG315 (18.5% vs. 12.8%), dan embB306 (16.5% vs. 6.1%). Hal ini tidak disebabkan oleh banyaknya galur Beijing pada pasien dengan riwayat pengobatan TB sebelumnya, diantara pasien baru pun mutasi pada gen penyebab resistensi berhubungan sangat kuat dengan genotip Beijing.

Percobaan binatang memperlihatkan bahwa M. tuberculosis genotip Beijing lebih virulen dibandingkan strain yang lain, tetapi data dari penelitian klinis masih kurang dapat memperlihatkan itu. Pada penelitian kohort prospektif dalam bab 5, kami menguji apakah genotip Beijing berhubungan dengan beratnya TB dan gagal pengobatan di Indonesia. Tidak ditemukan hubungan yang jelas antara genotip Beijing dengan manifestasi klinis atau kelainan pada gambaran radiologis paru, tetapi pasien yang sebelumnya terinfeksi oleh genotip Beijing, kultur sputum positif pada enam bulan setelah pengobatan dua kali lebih besar dibandingkan dengan pasien yang terinfeksi oleh genotip non-Beijing. Efek ini tidak disebabkan karena resistensi obat yang lebih tinggi pada galur Beijing. Hasil uji multipel regresi menunjukkan bahwa genotip Beijing merupakan faktor risiko independen untuk gagal pengobatan TB.

Setelah pengobatan TB, pasien masih mempunyai risiko yang tinggi untuk kematian, kambuh dan mengalami kerusakan paru yang permanen. Efek jangka panjang TB paru yang disebabkan oleh galur Beijing dan galur non-Beijing dibandingkan dalam bab 6. Seratus empat puluh enam mantan pasien TB dan 178 kontrol sehat yang sesuai dari sebuah penelitian kasus-kelola yang lalu diperiksa kembali setelah 1.5 sampai lima tahun kemudian. Mantan pasien TB yang pada awalnya terinfeksi oleh M. tuberculosis galur Beijing mempunyai risiko yang lebih tinggi untuk kambuh dibandingkan mantan penderita TB yang terinfeksi genotip lain. Kelainan fungsi paru lebih banyak ditemukan diantara 18 mantan pasien TB yang awalnya terinfeksi oleh M. tuberculosis genotip Beijing dibandingkan dengan 33 mantan pasien TB yang terinfeksi oleh genotip lain.


Distribusi geografis yang luas dari Mycobacterium tuberculosis genotip galur Beijing, dan kesamaan genetiknya mengesankan bahwa galur yang termasuk ke
dalam genotip ini mempunyai keuntungan selektif dibandingkan galur *M. tuberculosis* lainnya, seperti yang didukung oleh penelitian penelitian yang telah dipaparkan dalam bab 3-7. Di dalam bab 8 saya meninjau ulang seluruh penelitian epidemiologi molekuler, eksperimental dan klinik yang berhubungan dengan genotip Beijing yang sejauh ini telah dipublikasikan. Dari pustaka ilmiah, terlihat bahwa keberhasilan evolusi galur Beijing kemungkinan berhubungan dengan vaksinasi BCG, yang kelihatannya kurang bersifat protektif terhadap galur Beijing, dan juga berhubungan dengan resistensi atau resistensi ganda yang ditemukan di berbagai area. Sebagai tambahan, pada berbagai binatang percobaan terlihat bahwa galur Beijing lebih virulen, menyebabkan kerusakan histopatologi yang lebih luas, pertumbuhan dalam sel pejamu lebih meningkat dan lebih meningkatkan kematian. Pada tatanan molekuler, galur Beijing mempunyai sifat spesifik dalam hal struktur protein dan lemak serta interaksinya dengan sistem imun manusia. Disamping temuan bahwa genotip Beijing dikaitkan dengan polimorisme pada *SLCA1/NRAMP1*, galur Beijing juga telah dihubungkan dengan polimorisme di dalam gen pengkode TLR2, suatu reseptor pola pengenalan (pattern recognition receptor) yang penting untuk mikobakteria, mengesankan adanya kemungkinan ko-evolusi manusia-mikobakterial. Sebagai kesimpulan, genotip Beijing merupakan hasil evolusi dari *M. tuberculosis* yang menjadikannya lebih sulit untuk dikontrol baik melalui vaksinasi ataupun pengobatan dengan antibiotik.

**Pembahasan umum**

Berdasarkan berbagai penelitian yang telah diringkas di atas, dapat dibuat dua kesimpulan penting.

Pertama, struktur populasi dari *M. tuberculosis* di Indonesia sangat heterogen disertai dengan perbedaan geografis tetapi ditemukan predominansi galur yang berasal dari *clade* tunggal yaitu genotip Beijing. Hal ini secara jelas membuktikan bahwa *clade M. tuberculosis* ini mempunyai keuntungan selektif dibandingkan dengan *clade* yang lain. Kedua, beberapa faktor yang berlainan kemungkinan bertanggung jawab terhadap predominansi genotip galur Beijing, termasuk virulensi yang lebih tinggi (terutama tingginya persistensi bakteri ini selama pengobatan), lebih resisten obat, dan kemampuan adaptasinya terhadap sistem imun alamiah manusia.

Berikut, akan didiskusikan temuan temuan di atas secara lebih rinci terutama difokuskan ke dalam empat aspek; epidemiologi, karakteristik molekuler, fenotip klinis, dan hubungannya dengan sistem imun pejamu.

**Temuan utama pertama** dari tesis ini adalah tingginya prevalensi *M. tuberculosis* genotip Beijing, yang secara bermakna menyebabkan tingginya keragaman genetik *M. tuberculosis* di Indonesia. Keragaman genetik *M. tuberculosis* yang
tinggi ini berlawanan dengan hasil penelitian dari area lain yang mempunyai prevalensi tinggi termasuk Vietnam, yang hanya mempunyai dua genotip utama yaitu; Beijing (35-54%) dan EAI (20%) (13-16). *M. tuberculosis* genotip Beijing merupakan genotip yang paling banyak ditemukan di Indonesia (33%), dan proporsinya stabil selama periode penelitian ini yaitu dari tahun 2001 sampai 2006, serta sama dengan prevalensi yang tercatat pada penelitian yang lebih lama (32.4%) (17). Jadi, genotip Beijing kelihatannya merupakan galur endemik yang bersirkulasi di Indonesia.


**Temuan utama kedua** di dalam tesis ini adalah lebih tingginya prevalensi mutasi gen resistensi obat pada *M. tuberculosis* Beijing genotype strain dibandingkan dengan galur non-Beijing. Mutasi lebih banyak ditemukan pada gen *rpoB*, *katG315*.

Temuan utama ketiga di dalam tesis ini ialah pasien yang terinfeksi *M. tuberculosis* galur Beijing mempunyai risiko lebih tinggi untuk gagal pengobatan dan kerusakan paru jangka panjang. Hal ini bukan disebabkan oleh ketidakpatuhan makan obat atau adanya resistensi obat. Penelitian kami adalah yang pertama kali membuktikan bahwa genotip Beijing berhubungan dengan kultur sputum positif sesudah selesai pengobatan pada mereka yang tidak resisten obat. Temuan ini menunjukkan bahwa genotip ini mempunyai

Serupa dengan tingginya angka gagal pengobatan, pasien yang terinfeksi M. tuberculosis galur Beijing juga banyak yang menjadi kambuh dan mengalami kerusakan paru yang lebih lama. Orang bisa mengambil kesimpulan mengapa hal ini terjadi. Pertama, TB aktif tidak ter-eradikasi secara sempurna selama pengobatan pertama sehingga menyebabkan kambuhnya. Kedua, kerusakan karena episode TB aktif secara permanen telah merusak pertahanan lini pertama tubuh pejamu, oleh karena itu membuat mereka lebih rentan untuk kambuh karena re-infeksi. Akhirnya, genetik dan faktor lain yang mempengaruhi pertahanan tubuh alamiah pejamu dapat menjadi predisposisi seseorang untuk berkembang ke arah TB aktif baik setelah infeksi primer maupun setelah re-infeksi oleh M. tuberculosis galur Beijing.

Keberhasilan galur Beijing – suatu kerangka pemikiran:

Nilai tambah penelitian
Disamping pertanyaan pertanyaan spesifik di dalam tesis ini, ada dua nilai tambah pada penelitian ini. Pertama, temuan penelitian ini mempunyai implikasi khusus kepada kesehatan masyarakat dan penanggulangan TB. Kedua, kerangka teoritis dan pengalaman praktis yang menjadi basis dari tesis ini dapat membantu memutuskan cara mana yang paling efektif untuk mengimplementasikan atau meningkatkan *molecular typing* *M. tuberculosis* dan mikobakteriologi secara umum di Indonesia. Berikut ini akan saya fokuskan ke dalam dua nilai tambah penelitian ini yaitu; aspek kesehatan masyarakat dan aspek laboratorium.

**Aspek Kesehatan Masyarakat**
Tesis ini mengangkat beberapa permasalahan yang berhubungan dengan penanggulangan TB di Indonesia. Pertama, galur Beijing yang merupakan genotip terbanyak di Indonesia, berhubungan dengan penularan yang lebih tinggi dan
kasus gagal pengobatan. Genotip ini merupakan tambahan faktor risiko untuk kegagalan, setelah ko-infeksi HIV, diabetes, dan status nutrisi yang rendah dll. Pada setting penelitian ini angka drop out rendah dan kepatuhannya tinggi (S1).

Kedua, angka gagal pengobatan cukup tinggi dibandingkan dengan data dari negara negara yang menggunakan dua regimen awal yang terstandarisasi yang mempunyai rerata angka gagal pengobatan sekitar 5.0% (di negara negara yang mempunyai prevalensi MDR lebih dari 3%), atau 1.6% (di negara negara yang mempunyai prevalensi MDR kurang dari 3%) (S2). Disamping gagal pengobatan, kekambuhan juga kelihatannya cukup banyak: pada penelitian kami mengenai efek jangka panjang TB paru, 9.6% dari 146 mantan penderita TB mengalami kekambuhan.

Permasalahan tingginya gagal pengobatan dan tingginya angka penularan belum menjadi pertimbangan program nasional penanggulangan TB, karena program DOTS saat ini dianggap cukup sukses dengan >70% cakupan dan penurunan prevalensi TB. Karena galur Beijing dapat menjadi ancaman utama program penanggulangan TB, untuk memberantas kemunculan galur ini harus dilakukan berbagai upaya, misalnya kebutuhan akan dosis yang lebih tinggi atau perpanjangan masa pengobatan. Selain itu, identifikasi galur ini harus dipertimbangkan di tingkat masyarakat atau bahkan secara individual.

Ketiga, angka resistensi yang cukup bermakna ditemukan dalam penelitian ini. Walaupun sebagian disebabkan oleh bias seleksi, hal ini tetap merupakan permasalahan yang harus diatasi. Sejauh ini, Indonesia belum termasuk ke dalam survey internasional resistensi TB, juga tidak ada survey nasional, dan tidak ada satupun survey dengan jumlah dan kualitas yang memadai yang dipublikasikan. Pada kenyataannya hanya dua laboratorium yang memenuhi kontrol kualitas secara internasional. Kedua laboratorium ini menggunakan metode proporsional Canetti yang sudah berumur 50 tahun. Hampir tidak ada penelitian dan pengembangan yang dilakukan untuk uji resistensi obat TB di Indonesia. Hanya tahun ini program nasional TB mendukung upaya untuk mengevaluasi metode genotipik komersial sebagai uji skrin untuk resistensi TB. Beberapa tahun silam, dengan bantuan laboratorium rujukan TB Belanda (RIVM) metode 25-well plates dengan media Middlebrook yang transparan telah diperkenalkan (S3). Akurasi metode ini lebih baik, tetapi sulit untuk memproduksi plate yang berkesinambungan, karena kontaminasi selama produksi maupun inokulasi merupakan permasalahan yang besar. Sebagai alternatif, saat ini kami sedang mengevaluasi metode ‘in-house’ molekular yang murah untuk uji kepekaan obat, dan pemeriksaan ‘MODS’ (Microscopic Observation Drug Susceptibility) untuk uji resistensi TB, yang sangat ‘cost-effective’ dan hasilnya sangat baik di daerah yang kurang berkembang (S4, S5).

Kekurangan infrastruktur, sumberdaya manusia dan dana adalah rintangan untuk menuju uji kepekaan obat TB yang berkualitas tinggi di Indonesia.

Aspek teknik Laboratorium
Penerapan teknik molekuler di laboratorium klinik untuk pelayanan akan membantu deteksi cepat M. tuberculosis kompleks dan dapat berperan penting dalam meningkatkan kualitas uji kepekaan obat. Dalam penelitian ini kami menemukan bahwa pemeriksaan mikroskopik saja tidak cukup untuk deteksi TB karena kita akan kehilangan paling sedikit 17% kasus gagal pengobatan. Karena biakan memerlukan waktu yang lama, deteksi TB secara molekuler akan sangat berguna karena lebih cepat dan akurat. Walaupun tidak murah, metode ini dapat berperan di dalam penatalaksanaan kasus kasus sulit. Uji kepekaan obat metode molekuler bahkan memberikan dampak yang besar untuk penanggulangan TB. Walaupun tehnik ini akan sulit untuk diterapkan di semua laboratorium pelayanan di Indonesia, namun dapat dilaksanakan di laboratorium kesehatan masyarakat provinsi atau laboratorium rumah sakit rujukan yang saat ini sebagian sudah dilengkapi peralatan biomolekuler. Akan tetapi, laboratorium supranasional harus dibentuk lebih dahulu sebagai badan yang menjamin kualitas pemeriksaan deteksi molekuler. Bila kualitas metode yang rutin harus diperkuat di tingkatan Puskesmas, surveilans untuk mencari kualitas dari diagnosis awal dan resistensi obat seharusnya dilakukan di laboratorium rujukan provinsi. Selain itu, laboratorium rujukan seharusnya bekerjasama dengan fasilitas kesehatan yang menangani kasus kasus sulit misalnya putus beroat atau MDR-TB.

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Beberapa pertanyaan masih belum terjawab; 1) apa mekanisme yang mendasari virulensi yang berhubungan dengan galur Beijing? 2) bagaimana struktur populasi Mycobacterium tuberculosis kompleks di pulau pulau lain di Indonesia? 3) bagaimana gambaran klinis TB yang disebabkan oleh galur Beijing? 4) apa implikasi semua ini untuk penanggulangan TB di Indonesia? Permasalahan ini membutuhkan penelitian lebih lanjut.
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Penelitian yang sedang berlangsung di bawah kolaborasi ini adalah untuk deteksi molekuler M. tuberculosis, real time PCR untuk MDR-TB, MODS dll.yang ditujukan untuk pelayanan pasien. Harapan kami, di masa yang akan datang, kolaborasi ini dapat berkesinambungan dan terus melibatkan lebih banyak staf Fakultas Kedokteran Universitas Padjadjaran / R.S. Dr. Hasan Sadikin dan institusi-institusi yang terkait dengan keuntungan untuk semua pihak dan untuk tujuan penanggulangan TB lebih baik.

Kesimpulan
Tesis ini telah membantu mendirikan infrastruktur laboratorium klinik dan keterampilan metode molekuler untuk deteksi, identifikasi, uji resistensi dan epidemiologi M. tuberculosis kompleks. Semua ini memberikan sumbangan untuk meningkatkan pengertian mengenai mekanisme yang mendasari kemunculan M. tuberculosis galur Beijing. Penelitian komprehensif ini hanya dapat terlaksana melalui kerjasama yang baik antara berbagai peneliti dan institusi di Indonesia dan di Belanda. Masih banyak yang harus diteliti untuk memberantas TB secara efektif. Untuk itu saya berharap bahwa tesis ini dapat merupakan salah satu langkah dalam memahami TB dan dalam pengembangan alat diagnostik baru serta strategi untuk penanggulangan TB.
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About the Author
About the author

Ida Parwati was born on Wednesday, 29 December 1954 in Bandung, Indonesia. She completed her senior high school at S.M.A.N. 3 Bandung in 1973. She acquired her medical doctor from Faculty of Medicine Universitas Padjadjaran in 1982. Then she worked as a head of primary health care in Jambi province, central Sumatra, in Puskesmas Sarolangun (1983-1984) and Puskesmas Singkut (1984-1988). From 1989 to 1992 she conducted her clinical pathology training in Dr. Hasan Sadikin Hospital / FKUP, Bandung, followed by one year service at clinical laboratory of Majalaya District Hospital. Since 1995 she has become an academic staff member of the Department of Clinical Pathology in Division of Tropical and Infectious Disease / Clinical microbiology Division. She started her Strata 3 (PhD) at Postgraduate program Universitas Padjadjaran in 1999 and finished in October 2004 with scholarship from QUE project. In a meanwhile, started in 2001 she conducted tuberculosis molecular diagnostic and typing training in The Netherlands and started a PhD program in St Radboud University Medical Centre Nijmegen. Her activity now are related mostly to infectious disease field, working with general microbiology, conducting studies in tuberculosis, dengue and control of antibiotic resistant. She is an active member of the national TB program and has been appointed by the Ministry of health to become a trainer for Hospital Acquired Infection. She routinely published booklet for microbial and its susceptibility pattern at Dr. Hasan Sadikin Hospital every year since 2005.

Ida Parwati is married to T.M Pung Purnama, an obstetric-gynaecologist, and has two daughters Ratu Puri Paramita which has just graduated from Faculty of Medicine Universitas Padjadjaran and Ratu Purwanti which is now in the forth year of medical school in the same faculty.

She heads the Department of Clinical Pathology, Dr. Hasan Sadikin Hospital Faculty of Medicine Universitas Padjadjaran, focusing on improving the performance of the laboratory to acquire international standard accreditation.
International Publications


Gans J., van Crevel R. Adult meningitis in HIV-positive and -negative patients in Indonesia: a cohort study. AIDS (accepted for publication).


Factors Underlying the Success of the *Mycobacterium tuberculosis* Beijing Genotype in Indonesia

Ida Parwati