Circulating *Mycobacterium tuberculosis* strains in Vietnam

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Toward Viet Nam without tuberculosis

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ABBREVIATION

AFB	Acid-fast bacillus
AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacille Calmette-Guérin
CAS	Central-Asian
DOTS	Directly-Observed Treatment, Short-course
DST	Drug susceptibility testing
EAI	East-African-Indian
ECL	Enhanced chemiluminescence
HIV	Human Immunodeficiency Virus
IS*6110*	Insertion Sequence *6110*
IUATLD	International Union against Tuberculosis and Lung Disease
LAM	Latin-American-Mediterranean
LJ	Löwenstein-Jensen
MDGs	Millennium Development Goals
MDR	Multi drug resistance
M. tb	*Mycobacterium tuberculosis*
NHRD	National Hospital for Respiratory Diseases
NIHE	National Institute of Hygiene and Epidemiology
NTP	National TB control program
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
Spoligotyping	Spacer oligonucleotide typing
SPSS	Statistical Package for the Social Sciences
TB	Tuberculosis
WHO	World Health Organization
XDR	Extensively drug-resistant

DEFINITION

Tuberculosis incidence is the estimated number of new tuberculosis (TB) cases arising in one year per 100,000 population. All forms of TB are included, as are cases in people with HIV. Estimates of incidence for each country are derived using one or more of four approaches, depending on the available data:

1. incidence = case notifications / estimated proportion of cases detected
2. incidence = prevalence / duration of condition
3. incidence = annual risk of TB infection x Stýblo coefficient
4. incidence = deaths / proportion of incident cases that die.

Tuberculosis prevalence refers to the number of cases of TB (all forms) per 100,000 population at a given point in time. Estimates include cases of TB in people with HIV. The prevalence of TB is calculated from the estimates of incidence combined with assumptions about the duration of disease:

Prevalence = incidence x duration of the condition.

The duration of disease is assumed to vary according to whether the disease is smear-positive or not; whether the individual receives treatment in a DOTS program, a non-DOTS program, or is not treated at all; and whether the individual is infected with HIV.

Tuberculosis case notification rate is the percentage of estimated new infectious tuberculosis cases detected under the internationally recommended tuberculosis control strategy DOTS. The DOTS notification rate for new smear-positive cases is calculated by dividing the number of new smear-positive cases treated in DOTS programmes divided by the estimated number of incident smear-positive cases for the same year, expressed as a percentage.

Definition given by the World Health Organization

ABSTRACT

Study tittle: Circulating *Mycobacterium tuberculosis* strains in Viet Nam.

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Background: Viet Nam, the 12th highest TB burden country in the world, has not shown significant decrease in TB incidence rate over the past years, despite its model National TB Control Program (NTP) and its attainment of the global target since 1997. One of the possible explanations for this is the mathematical model used to capture the dynamics of TB epidemiology might be insufficient (e.g., the emergence of the more virulent strains – *Mycobacterium tuberculosis* (*M. tb*) Beijing genotype, drug resistance, HIV co-infection in young adults, the negligence of the strong influence of diagnostic delay, risk factors associated with demography. This study therefore aimed to further investigate the hypothesis that Viet Nam's TB epidemic can be explained by molecular characterizations, drug susceptibility patterns and their association with demographic factors.

Methods: 580 *M. tb* strains collected in 5 TB hospitals from 3 main regions of Viet Nam during 2009-2010 and 100 historic strains isolated during 1996-2008 were analyzed by Spoligotyping and identified genotype. Then, 252 Beijing strains assigned by Spoligotyping were further analyzed by IS*6110*-RFLP. The discriminatory of RFLP patterns was compared using BioNumerics software. The information from participants collected through questionnaires was statistically analyzed by SPSS software.

Results: *M. tb* population in Viet Nam was comprised of 19 different genotype families that assigned to 11 pooled families by Spoligotyping results. Beijing and East-African-Indian (EAI) were the most prevalent. Among 580 newly collected strains, 44.7% were Beijing genotype. This genotype was more prevalent among patients who had drug resistance, lived in urban area, of young age and are female. 238/252 of the Beijing strains were belonged to one group with high similarity of IS*6110*-RFLP patterns but not identical. 7/22 multi-drug resistance (MDR)-TB Beijing strains were identical to non-MDR-TB strains. The strain patterns from the North were diverse while that from the South were disseminated. The atypical Beijing RFLP patterns with low-copy of IS*6110* was found in 6/252 (2.4%) strains

Conclusion: The Beijing genotype has been predominating in the nation since 1996. However, its prevalence is declining from 54% in the 1998-1999 study to 44.7% in our study, while EAI family seems to be expanding. In Viet Nam, Beijing genotype is associated with patients who have urban residence, younger age, female gender, drug resistance but not with BCG vaccination history. One third of the MDR-TB strains were identical to non-MDR-TB strains demonstrating that MDR-TB had been acquired after transmission. The atypical Beijing patterns with low-copy of IS*6110* is tended to be eliminated from the *M. tb* population. The differences between the IS*6110*-RFLP strain patterns of the North and the South suggested that the *M. tb* Beijing populations of the two regions were possibly derived from different ancestor strains.

CHAPTER I: LITERATURE REVIEW

1.1. The global tuberculosis situation

1.1.1. Tuberculosis epidemic in history

Tuberculosis (TB) is an ancient-originated disease that has killed more persons than any other infectious disease (1). It is assumed to be presented in human population millions years ago (2;3). The members of modern *Mycobacterium tuberculosis* (*M.tb*) complex appear to have a common ancestor that is the successful survivor from a major evolutionary bottleneck 15,000-20,000 years ago (4). There is some evidence of *M.tb* progenitor's occurrence in humans several thousand years ago in a wide geographical distribution. It has been found in archaeological bones, mummies in Egypt, and remains located in the Eastern Mediterranean, pre-Columbian America and Europe (5).

The biggest TB epidemic in history of Western Europe and North American, known as the Great White Plague, started in the beginning of the 17th century and lasted for 200 years. By 1650, TB became the leading cause of death and was considered incurable. A rate of more than 1,000 TB cases per 100,000 population was seen in the late 1700s and early 1800s. During this time, most people were infected with TB. The mortality rate was estimated up to 25% (5). In a short time, TB killed 80,000 people in Marseilles, 70,000 in London, over one million in Germany and half of inhabitants in Italy (6). The high population density as well as the poor sanitary conditions of the most European and North American cities at that time provided an ideal environment for the wide spread of this airborne disease. The epidemic reached other parts of the world through European exploration and colonization (7).

Many efforts to cure the disease were made but mostly were ineffective. The first widely practiced TB treatment was the introduction of sanatoria in 1859, facilities where patients took bed-rest, ate healthy diets and performed exercises, while continuously being exposed to fresh air. At that time sanatoria were commonly found throughout Europe and the US and mostly built in remote or mountainous areas. They were helpful in assisting the recovery process of TB patients and concurrently isolating the source of infection from the general population. Beginning in 1880, surgical procedures were introduced and probably improved cure rates (7).

From March of 1882, the fight against tuberculosis was changed dramatically by discovery of the tubercle bacillus, the bacteria causing the disease. The bacilli were first described and isolated by the German scientist Robert Koch (1843-1910). When public health authorities realized that TB was contagious and preventable, they started a large community education campaign, including distribution of books, posters, stamps and advertisement. With increased knowledge of TB, together with better living conditions, good nutrition and isolated infection sources, TB mortality rates started to decline in the 19th century. Then, following the development of Bacille Calmette-Guérin (BCG) vaccine, the discovery of a series of new anti-TB drugs and the application of combined antibiotic chemotherapy method, TB was considered to be completely curable in the 20th century (7). There was optimism that TB would be eliminated.

1.1.2. The reemergence of tuberculosis

In developed countries, TB morbidity and mortality declined steadily during the 20th century due to the improvement in public health. The disease was highly controlled. Although it was never eliminated from the developed world, there was a period in which the TB epidemic was largely neglected and TB control programs almost vanished. In contrast, TB was devastating the developing world and became a serious threat to the community (8). From the mid of 1980s, TB rates began to increase again in Europe and the USA. The spread of human immunodeficiency virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) epidemic involving TB/HIV co-infection, the emergence of multi drug resistance (MDR), the increase in life expectancy, the rise of immigration from high TB prevalent countries and the decline in TB control program activities were responsible for this resurgence (9;10).

Two major factors drove this reemergence of TB. Firstly, the global spread of HIV/AIDS epidemic and high rate of TB/HIV co-infection greatly contributed to the rise of TB cases. Diagnosis of TB became more difficult in patients with HIV due to false-negative tuberculin skin tests, sputum smear-negative disease such as pulmonary and extra-pulmonary, and atypical radiological manifestations (10). Secondly, the TB program was neglected in the industrialized countries. Its inefficiency was demonstrated in a 1988 study which found that 89% of patients discharged from TB treatment were lost to follow-up and failed to complete therapy. Among

those, 27% returned to the hospital within a year with confirmed active TB (11). The non-compliance of patients and the misuse of drugs by health workers frequently resulted in the emergence of bacteria resistant to drugs in which MDR-TB were extremely difficult to treat.

In this new situation, the TB epidemic has increased again globally and millions of people die each year from the disease. The World Health Organization (WHO) declared TB to be a global health emergency in 1993. They promoted the Directly-Observed Treatment, Short-course (DOTS) strategy as the official policy in the fight against TB. The strategy was intended to enhance treatment supervision, including the regular taking of medication and completing treatment, and so to increase cure rates and reduce development of drug resistance (7).

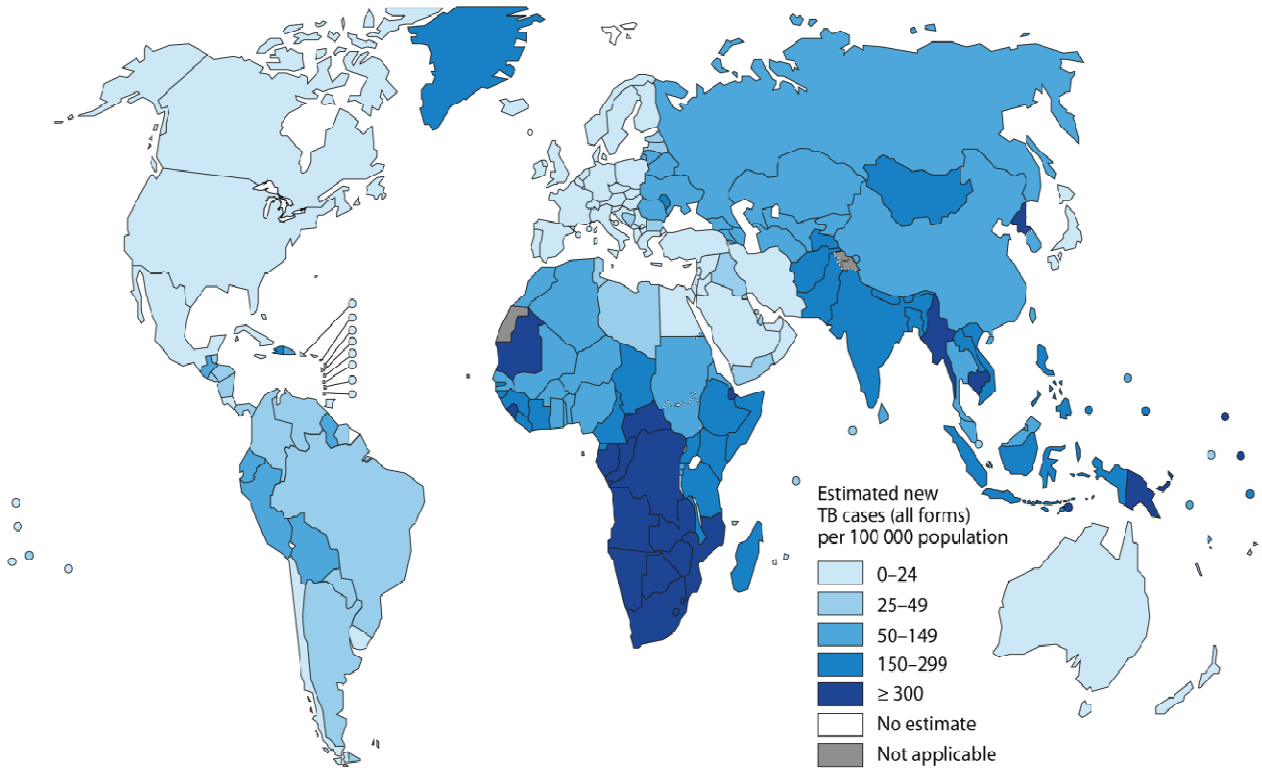


Figure 1.1: Estimated TB incidence rate globally, 2011. Source: WHO report 2012 (12).

1.1.3. The current tuberculosis epidemic

TB is the second leading cause of death from a single infectious agent worldwide, after the HIV. Currently, one-third of the world's population is latently infected with TB. Approximately 1.4 million deaths from TB occur per year in which 30.7% are among HIV-positive patients. The highest mortality rates are in Asia and Africa, with 32.9 and 26 deaths per 100,000 populations, respectively. The 22 high TB burden countries accounted for 83% of global mortality (Table 1.1) (12).

Table 1.1: Estimated tuberculosis incidence and mortality by regions from WHO, 2011 (12).

	Incidence			**Mortality** [1]		
WHO Region	No (thousands)	%	Per 100000 population	No (thousands)	%	Per 100000 population
Africa	2300	26	262	220	22	26
The Americas	260	3	28	21	2	2.2
Eastern Mediterranean	660	7.6	109	99	10	16
Europe	380	4.4	42	45	4.4	5
Asia [2]	5200	59	281	610	61.6	32.9
High-burden countries [3]	7100	82	163	820	83	19
Global	8700	100	125	990	100	14

(1)-Mortality excludes deaths among HIV-positive TB cases; (2)-Asia refers to the WHO regions of South-East Asia and the Western Pacific; (3) The 22 high-burden countries are Afghanistan, Bangladesh, Brazil, Cambodia, China, the Democratic Republic of the Congo, Ethiopia, India, Indonesia, Kenya, Mozambique, Myanmar, Nigeria, Pakistan, the Philippines, the Russian Federation, South Africa, Thailand, Uganda, the United Republic of Tanzania, Viet Nam and Zimbabwe.

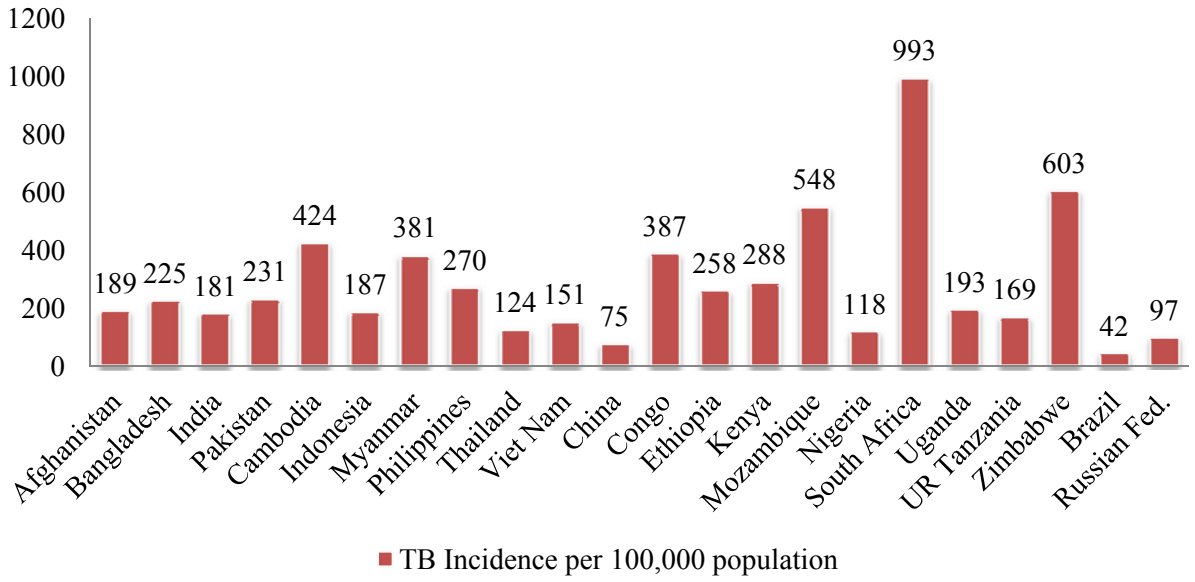


Figure 1.2: The TB incidence per 100,000 populations of 22 high-burden countries. The highest incidence rates were found in South Africa and other countries in Africa, Asia (12).

The World Health Organization (WHO) estimated that there were 8.7 million incident cases of active TB disease globally in 2011, equivalent to 125 cases per 100 000 population. In the line with mortality rate, the majority of new TB cases was in Asia (59%) and Africa (26%), smaller proportions of cases occurred in the Eastern Mediterranean Region (7.6%), the European Region (4.4%) and the Region of the Americas (3%). The 22 high-burden countries accounted for 82% of all estimated incident cases worldwide (Table 1.1, Figure 1.1, Figure 1.2) (12).

The global incidence rates were relatively stable from 1990 up to around 2001, and then started to fall (Figure 1.3). During the years 2010 and 2011, the rate of decline was 2.2%.

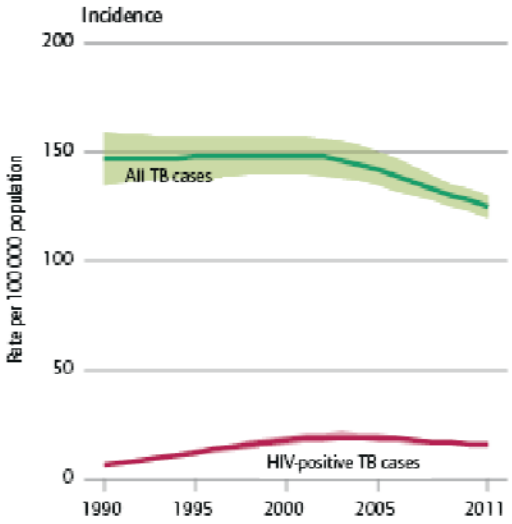


Figure 1.3: Global trends in estimated rates of TB incidence 1990-2011. Shaded areas represent uncertainty bands (12).

Furthermore, the emergence of multi-drug resistance (MDR) forms of TB, defined as resistance to at least the two most powerful drugs, Isoniazid (INH) and Rifampicin (RMP), is substantial, posed serious threat to global health. There was an estimated prevalence of 650,000 MDR-TB cases and 150,000 deaths annually (13). The MDR prevalence varies among countries, but is highest in the Russian Federation (12).

In addition, the MDR-TB primary infection in general population is quite high. About 3.7% of new TB patients in the world are infected with MDR strains. The rate of primary MDR-TB is highest in Europe region with 15% MDR among new patients (12). The WHO report on current global MDR-TB also demonstrated that *M. tb* is becoming more resistant. Among MDR-TB cases, 79% are resistant to at least three of the four 1st line anti-TB drugs and 9% develop further resistance to the 2nd line drugs, and are characterized as extensively drug-resistant TB (XDR-TB) (14).

The number of patients diagnosed and treated is small compared with the number of new and existing MDR-TB cases. It is estimated that only 10% of new MDR-TB cases are treated each year, and fewer than 2% of patients are receiving verifiable, quality-assured second-line anti-TB drugs (15). The person-to-person MDR-TB transmission in high-incidence countries is a serious problem. The risk of transmission is much higher when patients wait together for weeks in hospitals, many of which lack the proper infection control system. The failure to contain MDR-TB also reflects the delays in diagnosis, i.e. the inability to diagnose the problem quickly enough to prevent transmission while continuing to prescribe an ineffective standardized regimen (16).

1.2. Etiology of tuberculosis

Mycobacterium tuberculosis (*M. tb*) was first identified as the contagious agent causing TB by Robert Koch in 1882. The disease in human can also be caused by other members of *M. tb* complex, including *M. africanum*, *M. bovis*, *M. microti*, and *M. canetti.* Whereas *M. tb* and *M.africanum* mainly infect primates, *M. bovis* mainly infects cattle. Before the introduction of milk pasteurization, *M. bovis* caused many deaths by TB in Europe. Other *M. tb* complex members have rarely been documented (17).

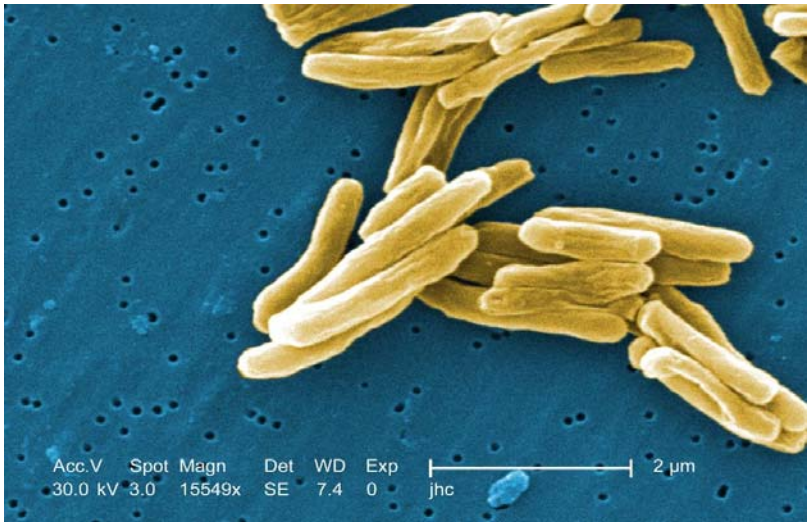


Figure 1.4: Shows the colorized scanning electron micrograph (SEM) of *M. tuberculosis* bacteria in magnification of 15549X. Photo Credit: Janice Carr, Centers for Disease Control and Prevention (CDC).

M. tb is an obligate intracellular pathogen which is aerobic, acid-fast, non-motile, non-encapsulated, non-spore and rod-shaped (Figure 1.4). It divides every 15–20 h, which is extremely slow growing in culture compared with other bacteria. This slow replication rate and ability to persist in a latent state result in the need for long durations of both drug therapy in TB patients and preventive therapy in people with *M. tb* infection (18).

Earlier studies on TB transmission performed by Wells and Riley in 1962 provided the first documented role of the droplets in the transmission of TB that set the basis for its containment. During coughing, sneezing, talking or spitting, people with active TB can eliminate large and small droplets containing viable bacteria. Large droplets tend to settle quickly onto the floor while smaller droplets (1-10 µm) remain suspended in the air for prolonged periods of time. The infection occurs through inhalation of aerosols containing bacteria produced by active TB patients. After infection, the initial responses usually happen in lungs by innate immune system such as macrophages. TB bacteria become active when the immune system fails to stop them from growing. Up to 10 % of exposed people develop the progressive TB disease soon after infection. Whereas, in most of the cases, TB bacteria are successfully controlled but still remain in a non-replicating or slowly replicating dormant state for the rest of the person's life. This infectious state, referred as latent TB infection, is clinically asymptomatic for years. They may

develop the disease from a latent infection later in life as a result of reactivation of dormant TB bacteria when their immune system becomes weak for some reasons (Figure 1.5) (7).

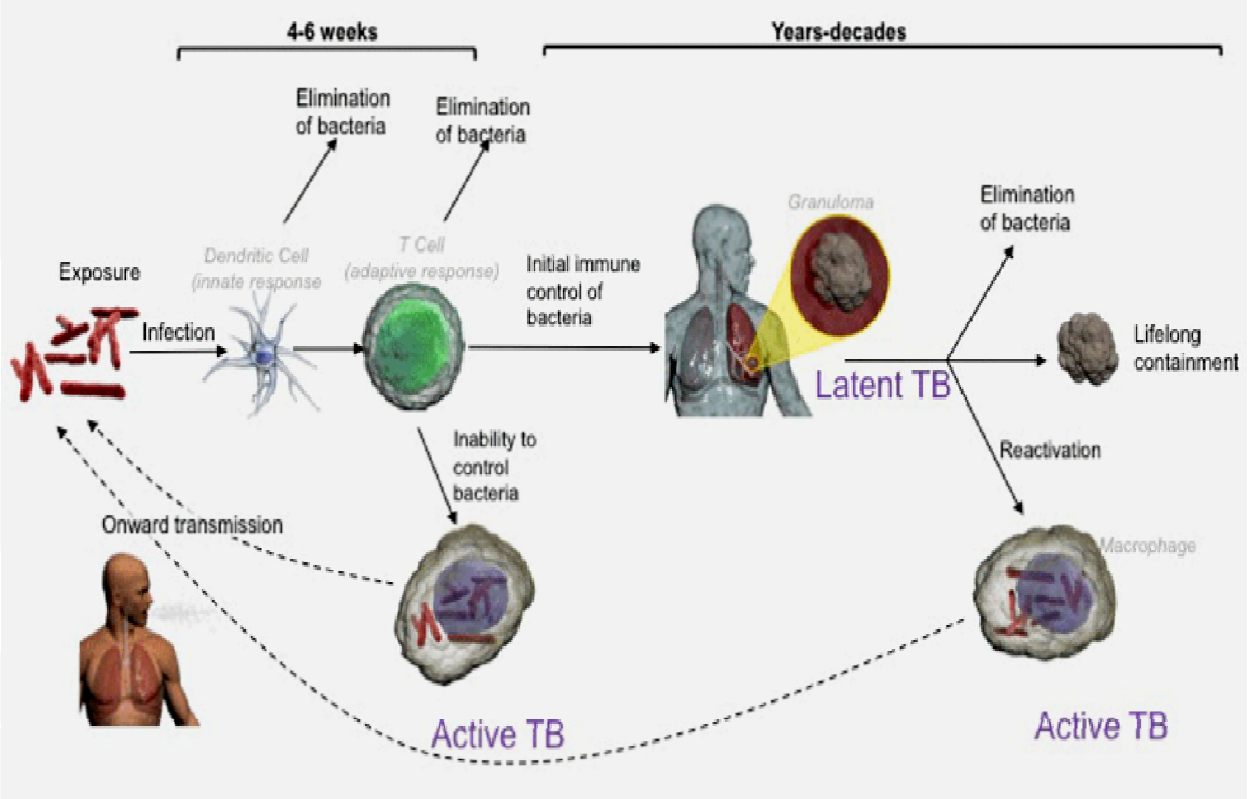


Figure 1.5: Natural history of Tuberculosis infection. Source: *infectionlandscapes.org*

1.3. Global tuberculosis control

1.3.1. Stop TB strategy

The burden of TB on society is massive. Even though effective drugs for treatment have been available for more than 50 years, yet every 15 seconds, someone in the world dies from TB. Approximately one person is newly infected with *M. tb* every second of every day. Left untreated, a person with active TB will infect an average of 10 to 15 other people every year (7).

After the declaration in 1993 that TB was a global public health emergency, WHO developed the DOTS strategy, which has become the internationally recommended approach to TB control. The strategy has 5 key components: comprising political commitment; diagnosis

using sputum smear microscopy; a regular supply of first-line anti-TB drugs; short-course chemotherapy with a standard system for recording; and reporting the number of cases detected by National TB Control Programs (NTPs) and the outcomes of treatment. The World Bank has ranked DOTS strategy as one of the "most cost-effective of all health interventions". A six-month supply of drugs for DOTS costs less than US$ 10 in some parts of the world (12).

After a decade of implementation, the new STOP TB Strategy and the Global Plan to Stop TB (2006–15) were launched in 2006 to address the important challenges that included the HIV-associated tuberculosis epidemic, the emergence of the MDR tuberculosis epidemic, weak health systems, and insufficient engagement with private healthcare providers and with communities. The new recommended strategy as part of the Millennium Development Goals (MDGs) is targeted to the falling in TB incidence, 50% decrease in global prevalence and death rates by 2015 compared to their levels in 1990 (18).

Up to now, almost all countries have adopted the strategy and progress is being monitored against some goals and targets. Between 1995 and 2011, 51 million people were successfully treated for TB in DOTS program (out of 60 million treated in total). This endeavor saved approximately 20 million lives (12). The earlier worldwide target of an 85% treatment success rate for sputum smear-positive cases was first achieved in 2007 and the MDG 6 target to reverse the rising incidence in TB incidence rates has been fulfilled since 2004. However, despite substantial progress in global TB control, it is unclear why TB incidence is only falling at a rate of less than 1% per year. Although the global case detection rate by DOTS programs increased almost linearly from 11% globally in 1995 to 28% in 2000 and accelerated to 56% in 2005, then stabilized at around 60% and currently at 67% in 2011, it is falling shortly under the 70% target. Moreover, the other target of halving the 1990 TB prevalence and mortality rates by 2015 is unlikely to be met worldwide due to the epidemics of TB/HIV co-infection in Africa and MDR-TB in Eastern Europe (11;15;16).

1.3.2. Vaccine for tuberculosis

Bacille Calmette-Guérin (BCG), still the only vaccine available against TB, was derived from an attenuated strain of *M. bovis* and first tested in humans in 1921. Hence, BCG vaccination was quickly implemented worldwide. The vaccine has been given to 4 billion people

and to more than 90% of the children in the world today, making it the most widely used vaccine worldwide (18). However, it has a minor impact on transmission of *M. tb* infection. Despite the evidence of protection against severe forms of childhood TB such as TB meningitis and miliary TB, BCG has been found to be of variable efficacy against pulmonary TB, ranging from 0 to 80% (19).

Since 1921, no new TB vaccine has been successfully developed. With the wide-spread of the disease and its consequences on the society, new globally more-effective vaccines are urgently needed for TB control and prevention. Recently, there are 12 vaccine trials that have progressed from the pre-clinical to the clinical phase aiming to new vaccine development or BCG efficacy improvement. But so far, none of them has reached the Phase III level of efficacy testing (19). Remarkably, one newly developed TB vaccine has been evaluated in Phase IIb trial (20).

1.3.3. Diagnostics of tuberculosis

Active TB is diagnosed by detecting *M. tb* bacteria in specimens from the respiratory tract for pulmonary TB or from other relevant body parts for extra-pulmonary TB. Although many new diagnostic methods have been developed, acid-fast bacilli (AFB) smear sputum microscopy and culture on Löwenstein-Jensen (LJ) medium are still the "gold standards" for diagnosis of active TB in low- and middle-income countries which account for 90% of the worldwide TB burden.

Despite AFB smear microscopy lacks sensitivity, especially in children and people living with HIV/AIDS, it is still useful to detect highly contagious patients because of its simplicity, rapidity (performed just in minutes) and low cost. In additional, its accuracy depends largely on the quality of the sputum specimen and the performance quality of the laboratory. Whereas culture needs long time (4-6 weeks), infrastructure and well-trained staffs to process, it is used to confirm smear microscopy results and to detect cases with low mycobacterial loads. It is also requested in cases at risk of drug-resistant TB for drug susceptibility testing (DST). Both methods are used to monitor the effectiveness of treatment by testing the presence of *M. tb* in patients' specimens and determining when they are less likely to be infectious (21).

Specimen for microbiology diagnosis

The success of diagnosis tests depends mostly on the quality of specimens. Poor quality of the sputum specimen often results in AFB smear microscopy and culture negative results. Therefore it is required to collect the sample properly, transport promptly and process carefully. For the diagnosis of pulmonary TB, first-morning sputum specimens (not saliva) obtained after a deep, productive cough on three different days are usually recommended. Satisfactory quality of sputum requires the presence of mucoid material and a volume of 3-5 ml. In the case of young children who cannot cough up phlegm, the gastric lavage is the procedure of choice. Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. When the transport or the processing is delayed, specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing (21).

AFB sputum smear microscopy

Smear staining is based on the high lipid content of *M. tb* cell wall which makes them resistant to decolorization by acid-alcohol after the primary staining. The specimen is spread onto a microscope slide, fixed, stained with a primary staining, de-colorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain a better differentiation between the bacteria and background. The slide is observed under the microscope for the detection of AFB then reported the results of AFB quantity (Figure 1.6, Table 1.2).

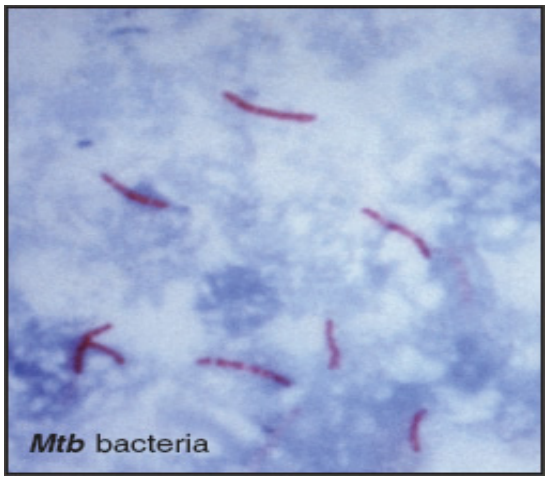


Figure 1.6: AFB sputum smear staining with positive result. Source: icfdn.org.

Table 1.2: Shown the quantity scale recommended by the World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease (IUATLD).

Count on Ziehl-Neelsen /Kinyoun stain (1000x)	Report
0	Non AFB observed
1-9/100 fields	Exact count
10-99/100 fields	1+
1-10/field	2+
> 10/field	3+

There are two methods using fuchsin in ethanol for primary staining, Ziehl-Neelsen and Kinyoun methods, thus making AFB appear red after de-colorization. Ziehl-Neelsen is a hot acid-fast stain because the slides are heat-fixed during incubation with fuchsin. In contrast, Kinyoun does not require heating referred as a cold acid-fast staining procedure.

Due to unreliable laboratory results, TB infectious patients may remain undetected, whereas people without the disease may receive treatment because of errors in AFB smear microscopy reading. Therefore, the quality assurances of AFB sputum smear microscopy as well as the quality of laboratory services need to be strengthened in National TB Control Programs (NTPs) of all countries which are relying mainly on this test for TB detection (21).

Culture

Smear microscopy is easy and quick, but it does not confirm TB diagnosis because other mycobacteria are also AFB in the smear microscopic result. Additionally, AFB microscopy positive results require a high bacterial load in the specimen. In contrast, culture technique confirms the diagnosis of active disease and detects a low load of bacteria, estimated 10-1,000 viable *M. tb* per ml of specimen. But most NTPs do not support its widespread use due to enhanced laboratory complexity, biohazard and cost (21).

Before culturing, it is necessary to decontaminate the specimens. Several methods have been used, most of them including the digestion of mucus or organic debris and elimination of micro-organisms. Both steps are done to maximize the probability of isolating *M. tb* in culture. The contamination rate should be kept between 3% and 5%. Rate lower than 3% may indicate

that the procedure used is too harsh and may be killing many bacteria. The most common methods used to decontaminate the sputum specimens are sodium hydroxide method; N-acetylcysteine-sodium hydroxide method and Ogawa - Kudoh method.

Different culture media are in use for the isolation of mycobacteria. The most common ones are based on egg and also contain high concentrations of malachite green to overcome the contamination with other bacteria. Of these egg-based media, Löwenstein-Jensen (LJ) and Ogawa are the most widely used. Generally, sediments are inoculated onto two LJ slants. Other species in *M. tb* complex different from *M. tb* such as *M. bovis, M. microti* and *M. africanum* often fail to grow on LJ medium. Because of the lack of a functioning pyruvate kinase enzyme, they are unable to use glycerol as a carbon source while LJ medium contains glycerol as the only available source of carbon (21).

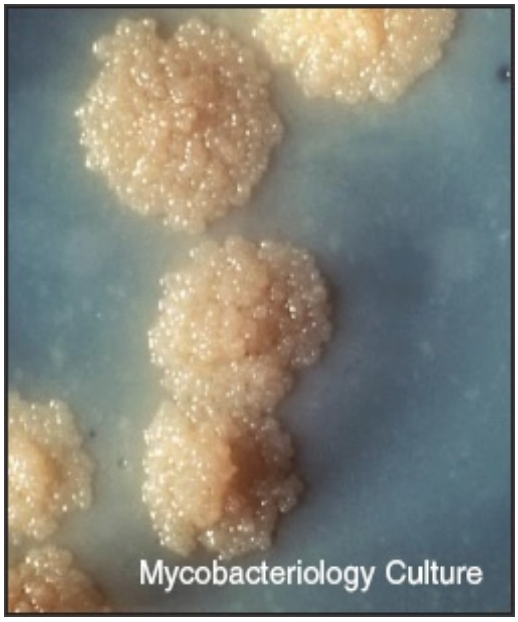


Figure 1.7: *M.tb* colonies growing in LJ medium. Source: icfdn.org.

New diagnostic methods

In 2007, automated liquid culture systems were recommended by WHO to be the gold standard for the TB diagnosis in low- and middle-income countries in combination with antigen-based species confirmation for diagnosis and drug susceptibility testing (DST). These systems are substantially faster and have a 10% greater yield than egg-based solid media. However, they are very expensive and prone to contamination. Alternative inexpensive noncommercial culture and DST methods such as microscopically observed drug susceptibility (MODS) and the nitrate reductase assay were endorsed by WHO in 2009 for use as an interim solution in resource-constrained settings. Despite many new methods having been developed, the conventional methods are still most widely used in these countries (18).

For the diagnosis of TB latent infection in the past century, tuberculin skin test has been the only screen available. However, it has failed to distinguish individuals infected with *M. tb* from individuals sensitised to other mycobacteria including *M. bovis* BCG vaccine. A decade ago, the interferon-γ release assays (IGRAs) were developed whereby interferon-γ titers were measured after in-vitro stimulation of peripheral blood mononuclear cells (PBMCs) with immune-dominant antigens expressed by members of the *M. tb* complex such as the 6-kDa early secretory antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10). These have now become the gold standard for identifying individuals whose immune system has previously encountered *M. tb*. There are two commercial methods that have been introduced and extensively tested even in individuals infected with HIV, the T-SPOT TB test (Oxford Immunotech, Abingdon, UK) and the QuantiFERON-TB Gold in tube (Cellestis Ltd, Carnegie, Australia) (18).

1.3.4. Treatment for tuberculosis

The WHO revised international guidelines for the treatment of TB in 2010 with more focus on controlling the growth of evidence base and escalating drug resistant problem worldwide. Earlier guidelines emphasized the use of two main standardized treatment regimens, one for new (previously untreated) cases and one for patients with sputum smear-positive disease who had previously received treatment (retreatment regimen). The drug combinations used in these two regimens differed only by the addition of a single drug which was incapable of preventing the emergence of drug resistance. Also, lack of laboratory infrastructure for culture and DST in many settings with a high burden of TB resulted in widespread empirical use of the retreatment regimen, thus inadvertently fuelling the emergence of MDR strains (18).

Pursuant to 2010 WHO TB treatment guidelines, Rifampicin should now be always given throughout the total 6 months of the first-line regimen. The current short-course treatment for completely eliminating active and dormant bacilli involves two phases, namely high-intensity and continuation phases. The initial high-intensity phase includes three or more drugs (usually Isoniazid, Rifampicin, Pyrazinamide and Ethambutol or Streptomycin), used for two months, which promote a rapid killing of actively dividing bacteria and result in the negativization of sputum. Then, the continuation phase is comprised of fewer drugs (usually Isoniazid and

Rifampicin) used for 4 or more months intended to kill any remaining or dormant bacilli and preventing recurrence (21).

Additionally, the crucial role of DST for guiding the individual management of retreated patients is also emphasized in the new guidelines because treatment for drug-resistant TB is very costly, prolonged, and toxic. However, the successful outcomes for MDR-TB are achievable in about two-thirds of patients while outcomes of XDR-TB are various (18). The proportion of XDR-TB patients who experienced favorable outcomes, defined as either cure or treatment completion, ranged from 18% to 67%, and the percentage of patients who died ranged from 0% to 50% (22). In most parts of the world, access to such therapy is very poor, with less than 2% of patients with MDR worldwide treated. Thus, in May 2009, the World Health Assembly passed a resolution urging the member states to provide universal access to diagnosis and treatment of MDR and XDR-TB (18).

1.4. Molecular epidemiology of tuberculosis

Molecular epidemiology is a field that has emerged largely from the integration of molecular biology into traditional epidemiologic research. Molecular methods help to refine data by providing more sensitive and specific measurements to facilitate epidemiologic activities and to give better understanding of disease pathogenesis at the molecular level (23). This approach has added a new dimension to the classical epidemiology of TB and greatly improved our knowledge about TB.

The development of molecular techniques to differentiate between strains of *M. tb*, DNA fingerprinting, during the last decades has unveiled the importance of strain variation in understanding TB pathogenesis, immune mechanisms, bacterial evolution and host adaptation in different population. It has lead to a profusion of studies in TB molecular epidemiology. These techniques have been applied at four major levels: in the laboratory, to optimize identification of cross-contamination events which can lead to a false diagnosis; in the patient, to determine whether recurrences are due to reactivations or exogenous reinfections; at the micro-population level, to identify clusters of cases infected by the same strains (recent transmission) and to differentiate them from orphan cases that are most probably due to reactivations; and at the macro-population level, to define the global distribution of *M. tb* lineages, to monitor the

international spread of high-risk strains, and to explore the evolutionary features of *M. tb* (24). Among the most commonly used genotyping methods, IS*6110*-based restriction fragment length polymorphism (RFLP) is considered as a gold standard and Spacer oligonucleotide typing (Spoligotyping) is the simplest technique for *M.tb* strain genotyping (25).

In different parts of the world, some families of *M. tb* strains have successfully adapted to specific human populations, which suggests the epidemiological strain diversity (26). For further analysis on genotyping, a continent and worldwide collection of strains has revealed 10 main *M.tb* families, identified by Spoligotyping method (Figure 1.8). Among those, the Beijing family reportedly predominates throughout eastern Asia with approximately 50% of the strains and spreads worldwide with a prevalence of 13%. The Central-Asian (CAS) family was found highest in Middle Asia. The East-African-Indian (EAI) family was more prevalent in Asia and Oceania. In Europe, the Haarlem family represents about 25% of the isolates. In South America, about 50% of the strains belong to the Latin-American-Mediterranean (LAM) family. The three families Haarlem, LAM, and T are the most frequent in Africa, Central America, Europe and South America. Outside Europe, The Haarlem strains were mainly found in Central America and Caribbean (about 25%), suggesting a link of Haarlem to the post-Columbus European colonization. The "ill-defined" T family, was found in all continents, and corresponded to about 30% of all strains (23).

Above all, the emergence of Beijing genotype continuously poses a serious threat to global TB control because of its high virulence and frequent association with MDR-TB. However, this association varies between different countries. This may be due to the heterogeneity in the adaptation and prevalence of the Beijing strains in the local population. Four patterns are found worldwide. Beijing family is epidemic and highly associated with drug resistance in Cuba, the former Soviet Union, Viet Nam and South Africa and at lower level in parts of Western Europe. In contrast, it is epidemic but drug sensitive in Malawi and Argentina. This family is endemic and not associated with drug resistance observed at high level in most of the East Asia and at lower level in parts of the United States. And there is very low level or absent of Beijing genotype in parts of Europe and Africa (27).

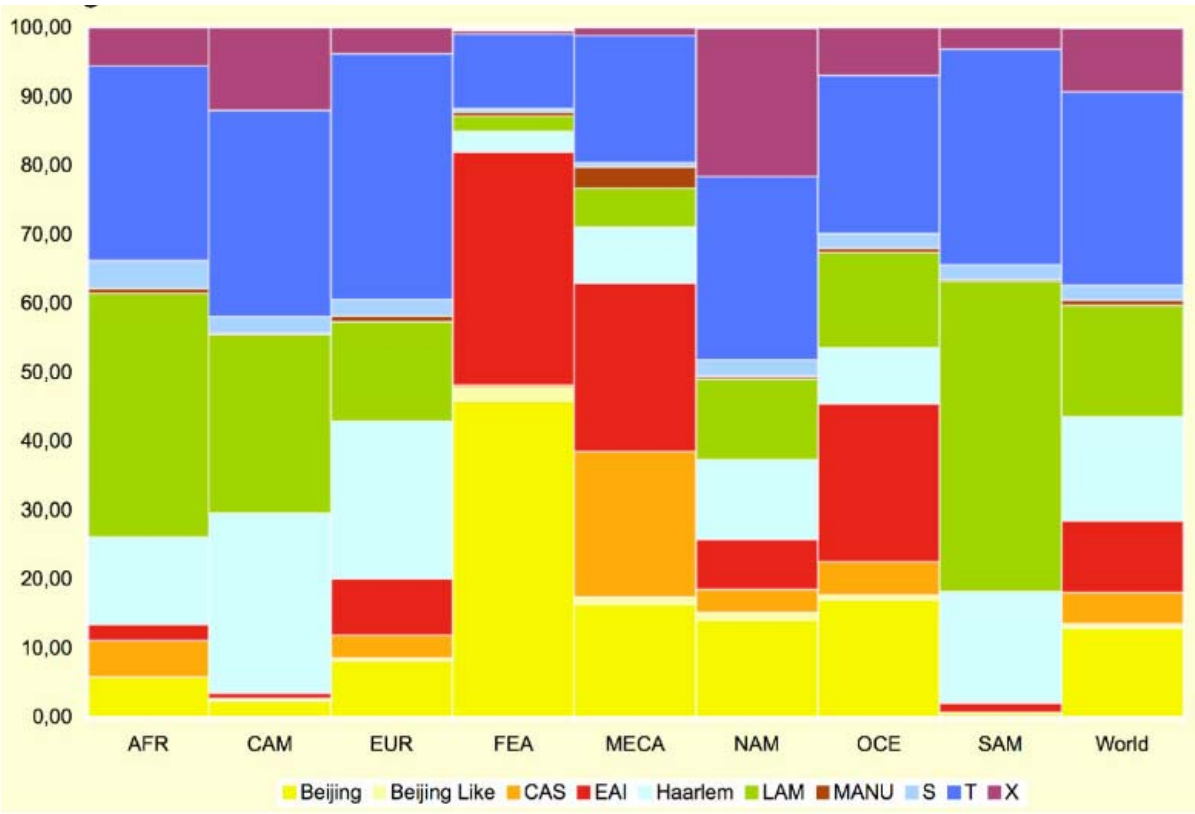


Figure 1.8: The global distribution of 10 main *M. tb* strain families, defined by Spoligotyping in SpolDB4 database (Beijing, Beijing-like, CAS, EAI, Haarlem, LAM, Manu, S, T and X families). The region abbreviations: AFR = Africa, CAM = Central America, EUR = Europe, FEA = Far-East Asia, MECA = Middle-East and Central Asia, NAM = North America, OCE = Oceania, SAM = South America (23).

Despite many new insights about TB obtained by several studies in molecular epidemiology, the remaining challenge is that most of studies were performed in the wealthy countries. The corresponding data from most high-burden areas remained limited because of poor infrastructure, lack of funding, the complexity of the techniques used and time required to interpret the results. The application of molecular epidemiology in support of NTPs in high incidence countries could undoubtedly be a useful research tool and help to enhance the current TB control strategies.

1.5. Country profile: Viet Nam

1.5.1. Background

Viet Nam is a South East Asian country with an area of 330,000 sq. km that stretches 3,260 km along the eastern coast of the Indochina peninsula. It borders China in the north and Laos and Cambodia in the west.

There are two different climatic zones in Viet Nam. Northern Viet Nam has monsoon climate with 4 distinct seasons, spring, summer, autumn and winter. Southern Viet Nam has tropical climate with 2 seasons, dry and raining.

The estimated total population of Viet Nam in 2011 was 87.8 million reported by General Statistics Office of Viet Nam. About 25.2% of the population was in the age group under 15 years, and 5.5% in the age group 65 years or more. The average population growth in 2011 was 1.1% (28).

There are 3 main regions in Viet Nam, the North, the South and Middle that have distinct characteristics as regards geographical situation, accessibility, population density and development. The largest city in the South is Ho Chi Minh City with a population of about 7.5 million and density of 3,580 people per sq. km. The capital Ha Noi, located in the North, has a population of about 6.7 million with a density of 2,000 people per sq. km (28).

Viet Nam belongs to the lower middle income countries with the Gross Domestic Product (GDP) growth at the rate of 5.8%. The Gross National Income (GNI) per capita was 1,270 USD in 2011 (29).

1.5.2. The National Tuberculosis Control Program

Since the reunification in 1975 following the war, the National Institute of Tuberculosis in Ha Noi has been responsible for the NTP. In 1986 the program adopted the TB control strategy of the International Union against Tuberculosis and Lung Disease (IUATLD) and WHO and began introduction of DOTS. Since the Government's declaration of TB control as a national priority in 1995, the DOTS program has expanded and is now in effect nationwide (30).

The TB control network encompasses all administrative levels including national, provincial, district and commune.

At the national level the director of the National Hospital for Respiratory Diseases (NHRD) in Ha Noi is responsible for the NTP. The Pham Ngoc Thach hospital in Ho Chi Minh City has been delegated for TB control in 22 southern provinces. Both hospitals are responsible for the overall implementation of the NTP in the provinces including training, drug distribution and supervision. They also act as reference laboratories and are responsible for the quality control of the laboratories at the peripheral levels.

At the provincial level, TB centers are part of the provincial general hospitals. The provincial TB centers are responsible for implementation of the TB program in the provinces. The provincial TB coordinator gives close guidance to the districts, supervises training activities, data collecting, and the distribution and proper use of drugs.

At the district level, the TB program is administered by TB units. Their tasks are to confirm the diagnosis by smear microscopy, to initiate the ambulatory treatment at communes near the patients' houses and to supervise the conduct of the NTP in the communes.

At the commune level, general staff is responsible for communicable diseases, including TB. Health workers are responsible for community health care, including TB in the villages. The commune and village levels identify and refer TB suspects to the districts and provide ambulatory treatment for TB (30).

1.5.3. Tuberculosis in Viet Nam

Viet Nam is the 12th highest TB burden country in the world and the 3rd in Western Pacific region, after China and the Philippines.

Table 1.3: Estimated TB burden of Viet Nam 2011 (12).

Population (million)	87.8
Mortality rate (excludes HIV+TB) / 100,000 population	21 (14-28)
Prevalence (includes HIV+TB) / 100,000 population	188 (153–227)
Incidence (includes HIV+TB) / 100,000 population	151 (112–197)
Incidence (HIV+TB only)	12 (8.9–16)
Case detection rate, all forms (%)	74 (57–100)
MDR in new TB cases (%)	2.7 (2-3.6)
MDR in retreatment cases (%)	19 (14-25)
TB patients with known HIV status (%)	59
HIV-positive TB patients (%)	8

The TB incidence was estimated 130,000 new cases in 2011 with the rate of 151 per 100,000 population. The case detection rate is 74% of all form. Among those detected, MDR-TB is estimated to occur in 2.7% in new cases and 19% in retreatment cases (Table 1.3). Only 2.9% of the 2010 estimated MDR-TB cases have been confirmed and have started treatment (12). The significance of this is that the risk of TB transmission is extremely high evidences a serious public health problem.

Since 1997, Viet Nam has achieved the global WHO targets of 70% detection rate and 85% cure rate in TB Control. At that time, Viet Nam was the only one of the 22 countries with the highest burden of TB worldwide to reach or exceed global WHO targets. It was considered as an example to other countries of a successful TB control program. The main reasons for success were the effective international partnerships combined with high political commitment of the government nationally and provincially as well as active participation of all organizations in the community (30).

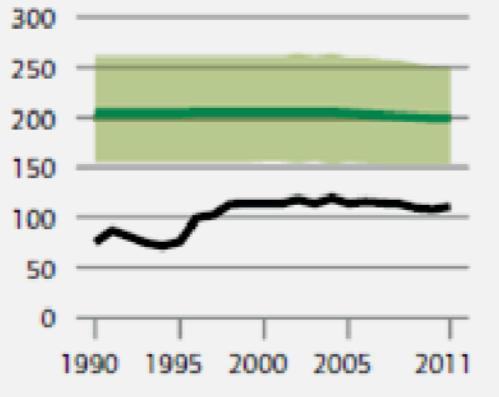


Figure 1.9: shows trends in case notification (all forms) (black) and estimated TB incidence rates (green) of Viet Nam during 1990-2011. Shaded areas represent uncertainty bands (12).

If the WHO targets of detection rate and cure were matched, the TB incidence of the country would decrease by 11% (range 8-12%) annually (31). However, this rate of Viet Nam has remained stable over the past years (Figure 1.9).

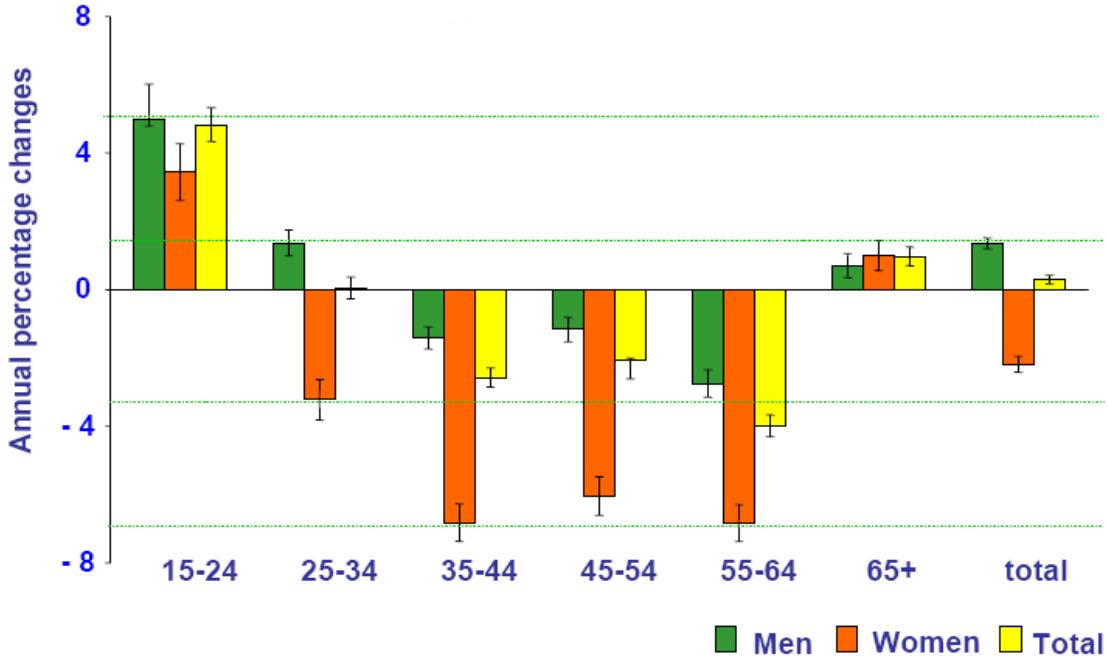


Figure 1.10: The annual percentage changes in notification rates during 1997-2004 by age and gender in Viet Nam (32).

There are several reasons that may contribute to the stability of TB rates. An emerging TB epidemic among young adults, particularly in men, causes concern because TB at younger ages tend to reflect recent transmission (Figure 1.10) (32). The incidence of TB patients with HIV infection has increased from the rate of 0.02 per 100,000 population in 1990 to the rate of 12 per 100,000 population in 2011 (12).

In addition, a number of studies have found a variety of *M. tb* strain families in Viet Nam which may reflect a diversity in virulence, transmission and drug resistance associated with specific strain genotype. An initial molecular epidemiology study conducted during 1994-1996 had found a significant difference of circulating strains between the North and the South of Viet Nam (33). They also found that drug resistance was more prevalent in the South than in the North. However, MDR-TB prevalence of the 2 regions was not significantly different. Among *M. tb* strain populations in Viet Nam, the Beijing strains were predominant both in the North and the South with a high rate of drug resistance. The Beijing genotype was found in 54% of new TB cases (34). The younger age (referred to active transmission) and drug resistance were factors associated with this genotype in Viet Nam (31;32) which perhaps led to the risk of increasing TB transmission in the community. In a study conducted in rural areas of Southern Viet Nam during 2003-2006, 85% of fluctuations in case notification rates between years were caused by the fluctuations in Beijing strain infections among the youngest group (35). Besides, this genotype was also a high risk factor for treatment failure and relapse (36) that undermined the effectiveness of TB control program.

Generally, there are three possible explanations for the static TB incidence rates, despite Viet Nam's model NTP and its attainment of the global target for TB control since 1997. First, the true case detection rate might be lower than estimated. Second, the true cure rate might be lower than reported. And third, the mathematical model used to capture the dynamics of TB epidemiology might be insufficient (e.g. the emergence of the more virulent strains – *M. tb* Beijing genotype, drug resistance, HIV co-infection in young adults, the negligence of the strong influence of diagnostic delay, risk factors associated with demography) (37).

Therefore, in order to find a solution for better strategies in TB control, the TB program in Viet Nam requires an overall picture of a TB epidemic influenced by both social and biological factors. The country lacks molecular epidemiological studies that access the *M.tb*

population structure representative for Viet Nam. It also urgently requires the latest updates regarding evaluating the *M. tb* Beijing genotype epidemic, the TB drug resistance situation and other associated risk factors.

Our study was conducted in this context and was intended to further investigate the hypothesis that Viet Nam's TB epidemic can be explained by molecular characterizations, drug susceptibility patterns and their association with demographic factors. This hypothesis was evaluated by studying the current *M. tb* population structure using molecular methods of DNA fingerprinting, Spoligotyping and IS*6110*-RFLP. Furthermore, the association between *M. tb* genotype and drug susceptibility patterns was analyzed with focus on the *M. tb* Beijing genotype. In addition, possible risk factors were statistically identified from demographic information given by TB patients through questionnaires. Outcomes of this project are helpful for better in-depth knowledge about circulating *M. tb* strains in TB epidemic of Viet Nam, as well as useful in strengthening strategies for TB control and treatment and hence possibly reveal new targets for intervention.

CHAPTER II: OBJECTIVES OF THE STUDY

Research question:

Can molecular characterizations and drug susceptibility patterns of *Mycobacterium tuberculosis* explain the TB epidemic in Viet Nam?

Primary objective:

To understand the TB epidemic in Viet Nam

Secondary objectives:

- To study the current *M. tb* population structure in Viet Nam by using DNA fingerprinting tools (Spoligotyping and IS*6110*-RFLP).

- To analyze the association between various *M. tb* genotype and drug susceptibility patterns with focus on Beijing genotype strains.

- To estimate measures of potential risk factors for recent TB transmission by using statistical analysis from information of TB patients such as demographic factors, co-morbidities (HIV), BCG vaccination history, delay in treatment.

CHAPTER III: MATERIALS AND METHODS

3.1. Study design: An analytic observational cross-sectional study

Analytic observational studies simply measures the exposure or treatments of the groups (38). A cross-sectional survey provides information about the frequency and characteristics of a disease in a "snapshot" of the defined population at a specific time. These studies suggest relationship between the diseases and other variables of interest. They are relatively cheap and simple to conduct. However, a single snapshot in time may not reflect the truth in the population. They cannot establish causality and directionality of the events. They also remain bias and confounders (39;40).

In this population-based molecular epidemiologic study of TB, our major objectives are to use molecular methods to investigate the *M.tb* population structure representative for Viet Nam, including identification of the predominant strains, evaluation of non-Beijing and Beijing strain patterns and suggestion of any relationship between risk factors and recent TB transmission. Due to the aim of this project, we have chosen the analytical observational cross-sectional design. It is cheaper and less time-consuming to conduct. Further it makes possible for us to have enough sample size that secure the representativeness of *M. tb* population structure in Viet Nam in a limited period of time and funding. Although not directly measured as in cohort study, the changes in the population structure of *M. tb* might be identified by looking at the distribution of genotypes in different age groups. If a certain genotype is more prevalent among young patients, this genotype may be associated with active transmission. If a genotype is predominantly prevalent among older patients, the genotype can be eliminated from the population. Subsequently, it will be investigated whether prevalent genotypes are associated with BCG vaccination (more prevalent among patients with a BCG scar), drug resistance or other factors such as co-morbidities (HIV), geography, gender, age, living condition.

3.2. Setting

The study was conducted in Viet Nam with the support of National Institute of Hygiene and Epidemiology (NIHE), Ha Noi and the National TB control program (NTP) in sample recruitments, data collection and analysis. Finances for the study were covered by the project "TBadapt", funded by European Committee (EC) and NIHE.

3.2.1. Study place

Due to the fact that Viet Nam has 3 main regions different in geography, economic and population density; we have integrated the local hospitals that represented for these 3 regions in patient recruitment and TB diagnosis. In total, 5 hospitals participated in the project: 3 from the North including 1 hospital in Thai Binh province and 2 hospitals in Ha Noi, the National Hospital of Respiratory Disease (NHRD) and Ha Noi Hospital of Tuberculosis and Lung Disease; 1 from the Middle area in Da Nang city; and 1 from the South, Pham Ngoc Thach hospital in Ho Chi Minh city. The sample recruitment took places in all the TB centers and units that these hospitals are responsible for.

3.2.2. Study population

Study population was recruited from all newly diagnosed patients admitted to the 5 TB local hospitals in 3 different parts of Viet Nam through the NTP system from December 2008 to July 2010. All new pulmonary TB patients that have chest X-ray diagnosis, sputum smear microscopy, positive *M. tb* culture and voluntary informed consent, were recruited in the study until July 2010 when the calculated sample size was attained. Smear-positive tuberculosis is usually rare in people younger than 15 years, and moreover it is difficult to collect sputum samples from children. Therefore the population eligible for the survey included all the residents aged more than 15 years who were present during recruiting time in the selected areas.

Our study population is only representative for culture-positive pulmonary TB patients who were registered for treatment by the NTP and has limitations for those who have chosen treatment at the private sectors, as well as false culture-negative and extra-pulmonary TB patients.

3.3. Sampling

Convenience sampling method was used in this study because it is fast, inexpensive and easy to conduct. But this method remains a source of bias that is the sample may not be representative. Therefore, in order to secure the representativeness, the NTP and TB local hospitals from Northern, Middle and Southern parts including both urban and rural areas of Viet Nam were in charge of recruitment.

3.3.1. Sample size calculation

Data from a previous study conducted in 1998 in Viet Nam shows that 54% of new TB patients were infected with Beijing genotype strains. They were slightly different between the North (58%) and South (53%) but results were not statistically significant (34). In another research conducted from 2003-2005 in southern part of Viet Nam, the Beijing strains were found in 33% new TB patients (32). Therefore, we assumed that the percentage of Beijing strain was appropriate at 43% during this study period.

For calculation of the sample size, we used Java Applets for Power and Sample Size software with Test of one proportion application (41). The actual p-value 43%, the significant statistic standard α 0.05 and the power 80% were chosen. By using these values, 400 new TB cases should have been recruited. We expected 10% lost from the sampling process. Consequently, the study aimed to include 440 TB patients.

3.3.2. Sample collection

All the newly diagnosed pulmonary TB patients, admitted to 5 TB hospitals in 3 parts of Viet Nam from 2009 to July 2010 that agreed to participate were recruited into the study. They had been given the information of the project, understood the main objectives of the study as well as the possible risk-benefit of participation and expressed their willingness to participate. The willingness to participate of the patients themselves was confirmed by their signature in the consent form.

Before registering for treatment at district hospitals, the patients were collected extra sputum sample, diagnosed with chest X-ray and interviewed about information on demography, BCG vaccination history. The participants produced at least 3ml of sputum and spited into a

sputum collected box with sealed lid which were then transported to provincial hospitals in the same day for culture. All positive culture *M. tb* strains were included in the study and performed drug susceptibility testing (DST).

In total, 580 *M. tb* isolates were sent to NIHE in Ha Noi for genotyping by molecular methods – DNA fingerprinting, together with the informed consent forms signed by the participants and the questionnaires for analysis. All the processes with regard to the participants' identification were performed anonymously by using the coding system.

3.4. Data collection

3.4.1. Interview

One interview form was used for all TB patients. The interview form was in Viet Namese language in a simple and easy-to-understand comprehension level. The pre-testing interview was conducted on 5 TB patients in order to test the data collection form before actually carrying it out in fieldwork. The aim was to check if they understand the question and give relevant answers during interview to minimize information bias.

The interview process was carried out at the local hospitals. Some health workers in NTP and local hospitals worked as our research assistants. They have received training on interview skills, details of the questionnaires and how to report what the respondent has answered. They first informed the TB patients that participation in the study was voluntary and explained more about the purpose of the study. Then, the participants were asked for informed consent and directly received an interview. The participants were also informed that the study will safeguard their confidentiality and anonymity.

3.4.2. Diagnostic tests

3.4.2.1. Chest X-ray

This method is commonly used for diagnosis on people who have suspicious TB infection. In active TB patients, the abnormality in the lung fields and lymph nodes are possibly seen in x-ray films. But it is difficult to decide the result even for experienced clinicians. Therefore, the chest X-ray may suggest TB infection but cannot confirm it.

3.4.2.2. Smear microscopy

This test is to examine the presence of acid-fast bacilli (AFB) in sputum samples produced by people who have TB symptoms by using Ziehl-Neelsen (ZN) staining technique. It is simple, cheap, performed just in minutes and gave good results without the need of special equipment. The method therefore can be done in TB units. It is still the main TB diagnostic and treatment monitoring method.

Smear microscopy was used in this study following Viet Nam NTP standard methods that based on the guidelines from IUALTD and WHO. Three sputum smears were collected for each patient. For out-patients, the first specimen was collected at the time symptoms suspected of TB are identified, this is commonly called a spot specimen. For hospitalized patients, specimens were obtained in the first 3 days in the early morning. This test was performed at provincial TB center and confirmed by the local TB hospitals.

The sputum was spread in the central area of the slide and air dried. The dried smear then was fixed on flame and proceeded staining with hot 0.3% Ziehl's carbol fuchsin solution for 5 minutes. Sputum smear appeared red in color. Decolorising was done by covering the smear with 25% sulphuric acid or acid alcohol solution (3% hydrochloric acid in 95% alcohol) for 3 minutes. This step can be repeated until the red color from the background disappeared. Counterstaining was done with 0.3% methylene blue solution for one minute. A properly stained smear by ZN showed bright red AFB against a light blue background (42).

Experienced and well-trained technicians were responsible for microscopic examination in each participated hospitals. They recorded and reported the results using IUATLD/WHO scale. For example, 10-99 AFB per 100 counting squares was presented as 1+ (42).

3.4.2.3. Culture

Bacteriological culture remains the gold standard test for diagnosis of active TB although it normally requires longer time and well-trained staffs to process. *M. tb* can be cultured from various specimens and in this study sputum was used. This technique confirmed the smear microscopy results by detecting bacilli and provided materials for further analysis such as drugs susceptibility testing and DNA fingerprinting.

The decontaminating N-Acetyl-L-Cystein-Sodium Hydroxide (NALC-NaOH) method and Lowenstein-Jensen (LJ) medium were used in all hospitals in this study following Viet Nam NTP standard methods that based on the guidelines from WHO.

The decontaminating agent NaOH is toxic for the contaminants and also tubercle bacilli. It can provide effective control of contaminants and also may over-kill the bacilli. Therefore, the mucolytic agent NALC, used for rapid digestion of sputum, enables the NaOH to be used at a low final concentration of 1%. Sodium citrate is included in the digestant mixture to bind the heavy metal ions which may be present in the specimen and could in-activate the acetyl-cysteine. The time needed to process 1 specimen is approximately 40 minutes (43).

The LJ medium was prepared slant into test tubes. Plastic Pasteur pipettes were used for primary cultivation with 2-4 drops (0.2-0.4ml) of the decontaminated sediment distributing over the surface. All cultures were incubated at 35°-37°C until growth was observed usually 3-4 weeks or discarded as negative after 8 weeks. The inoculated media was incubated in a slanted position for at least 24 hours to ensure even distribution of the inoculums (43).

Each positive culture sample was then collected into freezer tubes containing Tris-EDTA (TE) buffer and kept in freezer at -20^0C or -80^0C before transporting to NIHE Ha Noi for genotyping.

The cultures were done at provincial TB hospitals' laboratories except Thai Binh and Da Nang because they did not have well-equipment laboratory and well-trained staffs to perform the test. Therefore, all the sputum specimens from Thai Binh were transferred to Ha Noi Hospital of Tuberculosis and Lung Diseases and those from Da Nang to National Hospital for Tuberculosis and Respiratory Disease for culture.

3.4.2.4. Drug susceptibility test (DST)

DST means testing to find out which drugs the TB bacteria isolated from patients are sensitive to, and therefore whether the person has got drug resistant. The test helps to select efficient regiments to provide appropriate treatment for TB patients who have developed drug resistance.

Conventionally DST includes the *M. tb* culturing in the LJ media containing anti-TB drug. The observation of bacilli growth indicates drug resistance. In contrast, the inhibition of bacilli growth indicates drug susceptibility. The critical drug concentrations were used according to the WHO guideline as 0.2 µg/ml for Isoniazid, 40 µg/ml for Rifampicin, 2 µg/ml for Ethambutol and 4 µg/ml for Streptomycin (44).

However, this method has some disadvantages such as time consuming (up to 6 weeks), technical complexity, and good laboratory infrastructure requirement. Therefore, the test was performed only in 2 national reference laboratories at National Hospital of Respiratory Disease (NHRD) in Ha Noi and Pham Ngoc Thach hospital in the Ho Chi Minh City for quality assurance.

3.4.3. Genotyping tests

The most commonly used methods to differentiate between *M. tb* strains are Spoligotyping, the simplest technique (25), and IS*6110*-based restriction fragment length polymorphism (RFLP), regarded as a gold standard method (45). Both methods compare genotype of *M. tb* isolates in which fractions of its genome were arranged in various patterns.

3.4.3.1. Spoligotyping

Spacer oligonucleotide typing (Spoligotyping) is the most commonly used Polymerase chain reaction (PCR)-based technique for genotyping. This method is based on DNA polymorphism present at one particular chromosomal locus, the direct repeat (DR) sequence, in the *M. tb* genome. The DR region is composed of multiple direct variant repeats, which consist of a repetitive 36 base pair element and a short non-repetitive sequence (spacer) (21;43).

Using a set of PCR primers targeting the DR sequences, it is possible to amplify simultaneously all the region, including the unique non-repetitive sequences, or spacers, located among the DR. The presence or absence of the spacers is determined by using reverse hybridization on a membrane containing spacer oligonucleotides then visualizing on film as black squares after incubation with streptavidin-peroxidase and enhanced chemiluminescence (ECL) detection (Figure 3.1). Individual strains are distinguished by the number of spacers

absent from a complete spacer set that has been defined by sequencing this region from laboratory *M. tb* strains H37Rv and *Mycobacterium bovis* BCG (46).

The set of PCR primers used to amplify the whole DR regions are biotinylated DRa and DRb with the nucleotide sequences as following: DRa - biotinylated - 5' – GGT TTT GGG TCT GAC GAC – 3'; DRb - 5' – CCG AGA GGG GAC GGA AAC – 3'

Figure 3.1: An example of a Spoligotyping pattern. The membrane pattern was read from left to right in a row. The spacers were generated by DNA hybridization on the membrane and visualized on film as black squares after incubation with streptavidin-peroxidase and ECL detection.

Spoligotyping is widely used due to its low cost, high reproducibility and simplicity. This method is also very easy to interpret and computerize because of its binary result format (present/absent) and useful when comparing data bases from different geographical regions. It has been demonstrated to be helpful in discriminating between isolates of *M. tb* with low-copy-number of IS*6110* insertion (47). Another advantage of Spoligotyping is that, unlike IS*6110* genotyping, it can be performed with considerably less DNA (10ng) which makes it practical in acute clinical settings and less time-consuming (48).

Although Spoligotyping can be a powerful method to study the molecular epidemiology of *M. tb*, the discriminatory power of this method in general is inferior to that afforded by IS*6110*-RFLP and varies depending on the geographical area of the study. This method is less informative in regions with predominant or endemic strains such as Beijing genotype.

3.4.3.2. IS6110–RFLP

The method regarded as "gold standard" is based on a repetitive DNA element called insertion sequence *6110* (IS*6110*). The number of IS*6110* copies differs from 0 to over 25 and their insertion position in *M. tb* genome is variable between different strains. These sequences are not randomly distributed that makes possible to differentiate between strains (49). The *M. tb* strains are typed according to electrophoresis banding patterns of the copy numbers of this repetitive DNA element (Figure 3.2). As with any genotyping system, there are limitations inherent to IS*6110*-RFLP analysis. It needs approximately 4.5µg of bacterial DNA that required considerably huge amount of isolation and consequently sub-culture. Combined with a complexity of methods including many different techniques, it takes much long time to process (30-40 days). It also has limited resolution in analyzing strains with six or fewer copies of this insertion. Nonetheless, IS*6110*–RFLP patterns seem to be sufficiently stable (and polymorphic) for studying TB transmission dynamics at the local or population level and over time (25).

DNA extraction, PvuII restriction endonuclease digestion of DNA and Southern blotting were done following protocol of Dick van Soolingen et al. (50) and standardized method of Jan DA van Embden et al. (46) with some adjustments when performing in Viet Nam. After extraction, *M. tb* genomic DNA samples were measured the concentration. Amount of 4.5µg genomic DNA was digested by PvuII restriction enzyme for at least 4 hours. Agarose gels were loaded with 2µg of PvuII-digested genomic DNA and molecular size standard ladder DNA. The probe was amplified from purified DNA of *M. bovis* BCG strain by PCR using a set of IS*6110* targeting primers (Forward: 5'-CGT GAG GGC ATC GAG GTG GC-3'; Reverse: 5'-GCG TAG GCG TCG GTG ACA AA-3'). The 245bp fragment probe was then purified and peroxidase labeled.

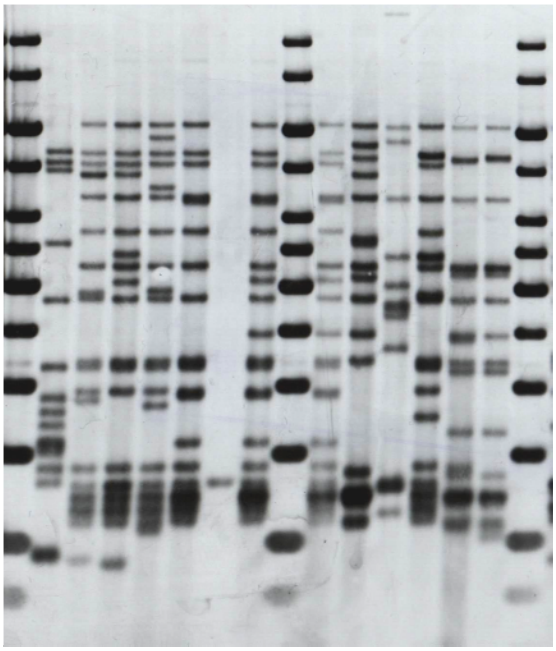


Figure 3.2: An example of IS*6110*-RFLP pattern. The polymorphisms were generated by variability in both the copy numbers and the chromosomal positions of IS*6110* (black bands) among *M. tb* strains.

3.5. Study variables

Dependent variables

The study had two dependent variables which were *M. tb* strain genotype and drug resistance. *M. tb* genotype was identified at the NIHE, Ha Noi. Drug resistance of *M. tb* strains was tested and confirmed by reference laboratories of NTP system which were at the National Hospital for Respiratory Disease (NHRD) in Ha Noi and Pham Ngoc Thach hospital in Ho Chi Minh City.

Independent variables

The independent variables in this study were regarded as the potential risk factors for infection of epidemic *M. tb* strains and/or drug resistant strains based on the literature review, including demographic factors, symptoms of TB, BCG vaccination history and HIV co-infection.

3.6. Data Analysis

The genotyping patterns of *M. tb* isolates were compared both by visual examination and computerized analyses. Spoligotyping patterns were identified by algorithm SPOTCLUST using database SpolDB4 model (available online at http://tbinsight.cs.rpi.edu/run_spotclust.html) (51). IS*6110*-RFLP patterns were characterized by BioNumerics software version 6.6 (Applied Math, Belgium) that is specialized for molecular classification. RFLP data was normalized according to standard molecular weight of DNA ladder bands. Latter, the band-based Dice method was used to cluster the RFLP patterns with 1% position tolerance in band matching.

All the information from participants collected through questionnaires was entered into a computer, together with laboratory diagnosed results and strain genotyping data. Then, the software SPSS version 18 (IBM, USA) was used for statistic analysis.

3.7. Study administration

In 5 hospitals cooperated in the projects, all the TB patient information and original data collection forms were transferred to the project administration office at the NIHE, Ha Noi. The data was encoded and computerized to ensure the anonymity and confidentiality of the patients.

All the data collection forms and signed consent forms of the participants were safely secured at the office. All the computerized data was also kept in a separate computer with password protection.

3.8. Ethical consideration

In a developing country as Viet Nam, where cultural, linguistic, economic, education and other barriers may prevail between researchers and subjects, it is especially important to ensure effective communication to obtain the informed consent. Informed consent is difficult to secure because the study itself is difficult to comprehend even for health professionals because of its molecular epidemiology approaches. Also, the socio-economic inequalities between researchers and subjects can result in subjects feeling that they have no choice when asked to participate in the study. A conflict of obligations may thus arise when the doctor is both the investigator and the provider of patient care. This conflict may be particularly complicated to resolve in developing countries where it may be impossible to separate the roles of investigator and career, and where highly inadequate health care resources and the pressures to enroll research subjects may overshadow concern for patients' best interests (52). These considerations need to be taken into account. More training is needed for the investigators on how to give information, how to empower the potential participants, etc…

In this study, confidentiality was protected by using the coding method for referring the participants. All the processes were done anonymously. The data with regard to social-demography factors from questionnaires and genetic *M. tb* population structure from molecular genotyping were entered by different researchers. Data once analyzed was kept and secured with password protection.

It was fair to say that there was "no-risk" seen from the point of view of participants if the coding system secures 100% anonymity and the given information does not create unjustified anxiety for participants. The study only collected their sputum samples and interviewed them once at the beginning stage. All the participants were given a small amount of money enough for travel reimbursement when participate in the project. Besides, at the individual level there was also "no-benefit". But the study was justified according to CIOMS Guidelines for review of epidemiological studies as it will undoubtedly benefit the group and community that the

participants represent. The project outcomes can access the overview picture of TB epidemic in recent years in Viet Nam by illustrating the *M. tb* population structure in association with drug resistance, age groups, genders, geography, living condition and other factors. It can help the Ministry of Health and NTP to form the better strategy that controls more effectively the emerging TB strains as well as the highly virulent and drug resistant strains, and so to reduce the TB transmission rate in community.

This study was a part of an internationally cooperated project "TBadapt" that have received ethical approval from the European Committee (EC). In Viet Nam, the study was first reviewed by the local Ethical Review Committee (ERC) at NIHE, then by ERC from Ministry of Health consisting of policy makers, healthcare system managers, professionals and senior researchers before getting the final approval to start the study in Viet Nam. Permission from NTP and local authorities were also obtained before recruiting participants.

3.9. Research Team

- Department of Immunology and Molecular Biology, NIHE, Ha Noi, Viet Nam: 5 persons.

  Responsibility:
  - Training interviewers, staffs in laboratory on molecular techniques used in the study
  - Regular follow-up with collaborated partners in Viet Nam, required progress reports and problem discussion
  - Administration and financial management
  - Coordinating the sample collection
  - Laboratory works: Performing Spoligotyping and IS*6110*-RFLP for all *M. tb* isolates from the North and Middle of Viet Nam
  - Data collection and analysis

- Department of Microbiology, National Hospital of Respiratory Disease (NHRD), Ha Noi, Northern Viet Nam: 4 persons

  Responsibility:
  - Recruiting participants, taking interviews, collecting sputum samples
  - Performing chest X-ray examination, smear microcopy, culture and DST

- Performing culture and DST for samples from Da Nang city
- Performing DST for samples from Thai Binh province
- Transferring *M. tb* isolates, interview forms and diagnostic test results to NIHE

- Ha Noi Hospital of Tuberculosis and Lung Disease, Ha Noi, Northern Viet Nam: 4 persons

  Responsibility:
  - Recruiting participants, taking interviews, collecting sputum samples
  - Performing chest X-ray, smear microcopy, culture and drug susceptibility test (DST)
  - Performing culture for samples from Thai Binh province
  - Transferring M.tb isolates, interview forms and diagnostic test results to NIHE

- Thai Binh Health Department, Thai Binh province, Northern Viet Nam: 1 person

  Responsibility:
  - Recruiting patients in Thai Binh Hospital of Tuberculosis and Lung Disease, taking interviews and collecting sputum samples
  - Performing chest X-ray examination and smear microcopy
  - Transferring sputum samples to Ha Noi Hospital of Tuberculosis and Lung Disease
  - Transferring interview forms to NIHE
- Da Nang Hospital of Tuberculosis and Lung Disease, Middle Viet Nam: 1 person

  Responsibility:
  - Recruiting patients, taking interviews, collecting sputum samples
  - Performing chest X-ray and smear microcopy,
  - Transferring sputum samples and interview forms to NHRD

- Department of Microbiology, Pham Ngoc Thach Hospital, Ho Chi Minh City, Southern Viet Nam: 5 persons

  Responsibility:
  - Recruiting participants, taking interviews, collecting sputum samples
  - Performing chest X-ray examination, smear microcopy, culture and DST
  - Laboratory works:Performing Spoligotyping and IS*6110*-RFLP for all samples from the South
  - Transferring *M. tb* isolates, interview forms and test results to NIHE

3.10. Time table

September – December 2008	- Visit and work with NTP and 5 partner hospitals - Recruit researcher assistants - Train interviewers - Train laboratory technicians - Conduct the pre-testing. - Modify the questionnaire as necessary
2009 –July 2010	- Patient recruitment - Data collection - Culture and isolation of *M. tb* strains from sputum samples - Perform DST of *M. tb* isolates - List and order materials and equipments needed for performing RFLP and Spoligotyping - Set up RFLP and Spoligotyping at the lab of Immunology, Department of Immunology and Molecular Biology, NIHE, Ha Noi
August 2010 – August 2011	- Culture and isolation of *M. tb* strains from sputum samples - Perform DST of *M. tb* isolates - Transfer consent forms, questionnaires and *M. tb* isolates from 5 partner hospitals to NIHE - Re-culture *M. tb* isolates to enrich DNA for further analysis (RFLP) - Data collection and entry

June - September 2012	- Work with the lab of Immunology, Department of Immunology and Molecular Biology, NIHE - Work with NIHE Board of Managers and Head(s) of the relevant departments - Work with NTP and 5 partner hospitals - Order new materials for 2 genotyping methods
October – December 2012	- Perform DNA extraction for all *M. tb* isolates - Train laboratory technicians with Spoligotyping and RFLP - Perform Spoligotyping on *M. tb* isolates
January – March 2013	- Perform Spoligotyping on *M. tb* isolates - Computerize data - Perform RFLP on strains identified as Beijing genotype by Spoligotyping
March – June 2013	- Get training on BioNumeric software - Enter data into the software - Data analysis by SPOTCLUST, BioNumeric and SPSS - Write thesis - Defend thesis

CHAPTER IV: RESULTS

4.1. Demographic characteristics of study population

During the period 2009 to July 2010, a total of 580 sputum samples were collected from new suspected pulmonary TB patients that were admitted to 5 TB hospitals in Viet Nam. There were 341 samples (58.8%) collected from the North including 196 samples from 2 TB hospitals in Ha Noi and 145 samples from Thai Binh Province. Fewer samples were collected in the Middle and the South, respectively 72 (11.9%) and 132 (29.3%) samples.

Table 4.1: Sample Distribution

		Number of samples	Percentage
Year	2009	275	47.4%
	2010	305	52.6%
Region	North	341	58.8%
	Middle	69	11.9%
	South	170	29.3%
Residence	Urban	317	54.7%
	Rural	259	44.7%
	No information	4	0.7%
Gender	Female	138	23.8%
	Male	438	75.5%
	No information	4	0.7%
Age	<25	67	11.6%
	25-34	132	22.8%
	35-44	136	23.4%
	45-54	113	19.5%
	55-64	61	10.5%
	65+	62	10.7%
	No information	9	1.6%

The study population was divided relatively equally into 54.7% living in cities and 44.7% living in rural areas. The gender distribution was large, with male patients constituting 3 times the number of female patients. There was some diversity in age distribution although we observed the expected distribution. Most patients were young adults, frequently in the age groups 25-34 and 35-44 (Table 4.1).

4.1.1. Sample distribution by region

In the North, more TB patients lived in rural areas (65.2%) than in urban areas (34.8%). In contrast, the majority of patients in the South (91.8%) and Middle (61.4%) were from urban places (Table 4.2).

Male domination of the study population occurred in all 3 regions (Table 4.2). The sex ratios were 3.54, 2.29 and 2.95 in North, Middle and South respectively.

Table 4.2: Living area and gender distribution by region

		North	**Middle**	**South**
Residence	Urban	118/339 (34.8%)	43/69 (64.2%)	156/170 (91.8%)
	Rural	221/339 (65.2%)	24/69 (35.8%)	14/170 (8.2%)
Gender	Male	263/339 (78.0%)	48/69 (69.6%)	127/170 (74.7%)
	Female	74/339 (22%)	21/69 (30.4%)	43/170 (25.3%)

In rural settings, most female (43/50) and male (117/230) patients were from the North, while only 4 female and 10 male were from the South, and 3 female and 21 male from the Middle. In contrast, Southern part had the highest number of female (39/87) and male (117/230) patients in urban areas, compared to the North (30 female and 88 male patients) and Middle (18 female and 25 male patients) (Figure 4.1).

The average study population age was 42.5, distributed in a range from 15 to 87. There was no significant difference of age distribution by regions, although there was a slight regional difference in the median age of 43.9, 41.9 and 39.9 respectively in North, Middle and South.

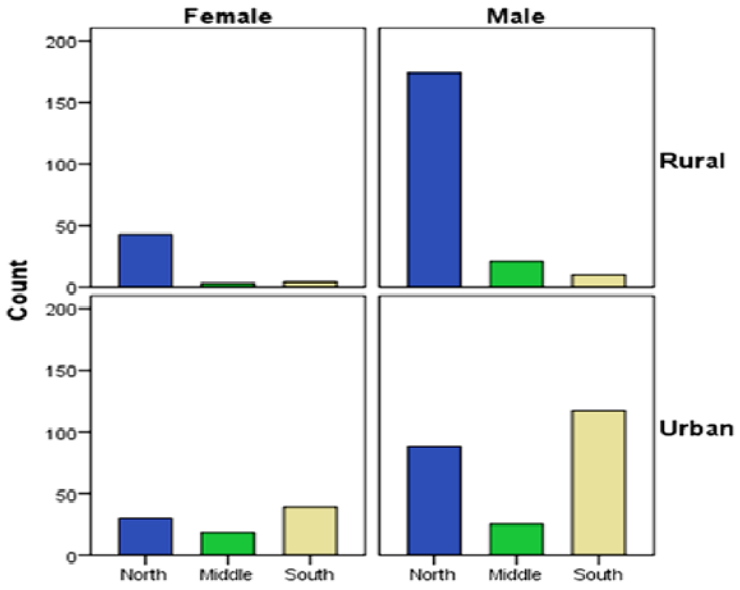


Figure 4.1: Gender distribution by residence in 3 regions of Viet Nam. In urban settings, more female and male patients came from Southern part of Viet Nam, whereas in rural settings most of female and male were from the North.

4.1.2. Age distribution by gender

Most male patients were recruited from the age groups 25-34, 35-44 and 45-54. The female patients were younger and belonged to the groups less than 25, 25-34 and 45-54.

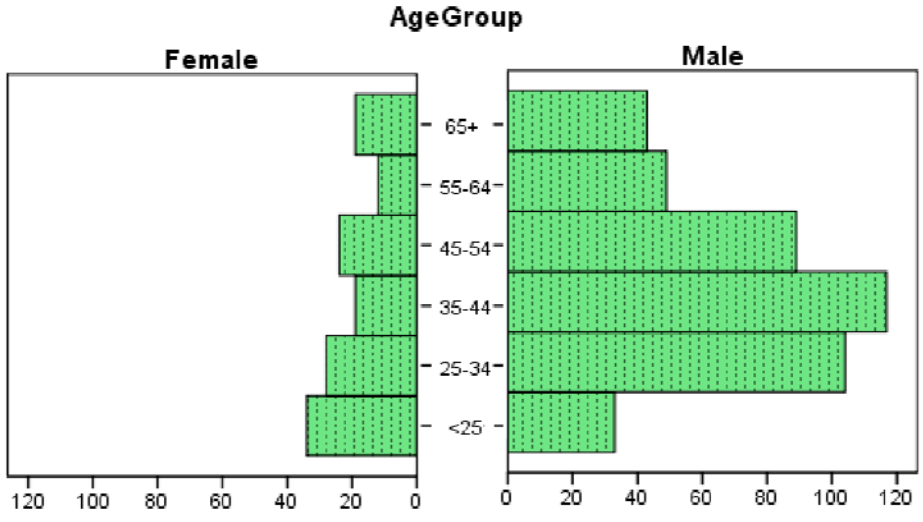


Figure 4.2: Age and gender distribution of TB patients, demonstrated that the majority of male patients was in the age-groups of 25 -54, while female patients were fewer and of younger age.

4.2. Clinical characteristics of study population

The information about BCG vaccination-history of TB patients was retrieved through scar inspection and interview. 12.4% (72/580) of the patients were vaccinated, among which 7.9% (46/580) had BCG scars and in 4.5% (26/580) scars could not be detected (Table 4.3).

HIV tests were done to all the patients. The results showed that 40/545 (7.3%) patients were confirmed HIV positive.

Sputum smear microscopy was performed at the TB centers or hospitals from 3 regions of Viet Nam. Microscopy result information was missing for 217/580 patients. Among the 363 patients where results were available, 89.5% (325/363) were positive and 10.5% (38/363) were negative. All the samples eligible to be included in the study were positive for *M. tb* culture. Therefore, 38 samples negative by microscopy were false negative. This false negative results were observed mostly from the North (23/38), some from Middle (15/38) and none from the South of Viet Nam. The differences in microscopy results by regions were statistically significant ($p<0.05$).

Table 4.3: Clinical characteristics of study population

		Number of samples	**Percentage**
BCG history	Vaccinated	72	12.4%
580	Unvaccinated	67	11.6%
	Uncertain	441	76.0%
HIV status	Positive	40	6.9%
	Negative	540	93.1%
Sputum smear	Positive	325	56.0%
	Negative	38	6.6%
	Information missing	217	37.4%

Among 245/580 patients reported to have symptoms indicative of TB prior to diagnosis, 90.4% had sputum produced by cough; 51.2% had fever; 45% had weight-loss; 29.7% had chest pain; 25.8% had loss of appetite; 22% had fatigue and weakness and 14.8% had shortness of breath as chief complaint. They normally sought treatment after some period of symptom-debut. Most participants reported that symptoms had been present for 1-3 months (44.9%) and 1-4 weeks (35.5%). Patients entering hospital after having symptoms for less than one week, 3-6 months, 6-12 months and more than 1 year were respectively 6.5%, 10.2%, 2.4% and 0.4%.

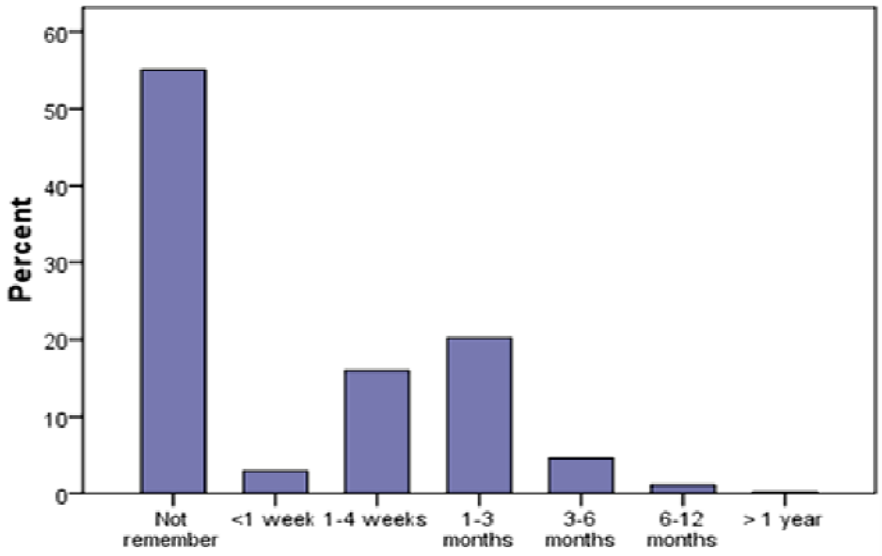


Figure 4.3: shows the delay time in seeking for treatment of TB patients. Most of them did not remember the periods after symptom-debut. Among patients remembering their initial period of symptoms, more were present in hospitals within 3 months.

4.3. Drug susceptibility patterns of *M.tuberculosis* strains

A total of 479/580 strains were tested for Isoniazid, Rifampicin, Streptomycin and Ethambutol susceptibility. The results are presented in Table 4.4. The prevalence of drug resistance was highest for Isoniazid and Streptomycin with 107 (22.3%) and 112 (23.4%) strains respectively, followed by Rifampicin and Ethambutol with 31 (6.5%) and 20 (4.2%) strains respectively. Multidrug resistant (MDR) was found in 3.8% (18/479) of all *M. tb* isolates.

The drug susceptibility by geographical area is presented in Table 4.4. There were minor insignificant ($p>0.05$) differences between the geographic regions.

Table 4.4: Drug susceptibility of 479 *M. tb* isolates from 3 regions in Viet Nam.

	H- DR	R-DR	S-DR	E-DR	Any DR	MDR
North	86/292 (29.5%)	26/292 (8.9%)	71/292 (24.3%)	17/292 (5.8%)	125/292 (42.8%)	15/292 (5.1%)
Middle	1/55 (1.8%)	0/55 (0%)	9/55 (16.4%)	0/55 (0%)	9/55 (16.4%)	0/55 (0%)
South	20/132 (15.2%)	5/132 (3.8%)	32/132 (24.2%)	3/132 (2.3%)	37/132 (28.0%)	3/132 (2.3%)
Total	107/479 (22.3%)	31/479 (6.5%)	112/479 (23.4%)	20/479 (4.2%)	171/479 (35.7%)	18/479 (3.8%)

Abbreviation: H-DR = resistance to Isoniazid, R-DR = resistance to Rifampicin, S-DR = resistance to Streptomycin, E-DR = resistance to Ethambutol and MDR = multidrug resistance (resistance to at least Isoniazid and Rifampicin)

MDR-TB was more prevalent in urban than in rural areas, but the difference was not statistically significant.

We found a correlation between MDR-TB prevalence and the delay time in seeking treatment of TB patients. The risk of being diagnosed with MDR-TB was 6.6 (95%CI: 1.5-30.0, $p<0.05$) times higher among people who came late to the hospitals, having had symptoms for more than 3 months, compared to those who sought treatment early, and had experienced symptoms for less than 3 months (Table 4.5).

There was no association of MDR-TB with other factors such as gender, age, HIV status, BCG vaccination history.

Table 4.5: The univariable and multivariable analysis of factors associated with MDR among new TB patients in Viet Nam.

Risk factors	MDR[1] Proportion	Odd ratio (95% CI[2]) Crude OR	Odd ratio (95% CI[2]) Adjusted[3] OR
Residence		(p>0.05)	(p>0.05)
Rural	4/212 (1.9%)	1	1
Urban	14/264 (5.3%)	2.9	2.9 (0.9-9.4)
Gender		(p>0.05)	(p>0.05)
Female	6/117 5.1%	1.6 (0.6-4.2)	1.6 (0.5-4.7)
Male	12/358 (3.4%)	1	1
Age group		(p>0.05)	(p>0.05)
<25	1/57 (1.8%)	0.3 (0.03-2.9)	0.2 (0.02-2.2)
25-34	5/100 (5%)	0.9 (0.2-3.7)	0.7 (0.1-3.2)
35-44	5/116 (4.3%)	0.7 (0.2-3.2)	0.7 (0.1-3.1)
45-54	1/96 (1%)	0.2 (0.02-1.7)	0.1 (0.01-1.5)
55-64	2/50 (4%)	07 (0.1-4.2)	0.6 (0.09-3.9)
65+	3/52 (5.8%)	1	1
Time to seek for treatment		(**p<0.05**)	(**p<0.05**)
Less than 3 months	4/145 (2.8%)	1	1
More than 3 months	4/26 (15.4%)	6.4 (1.5-27.5)	6.6 (1.5-30.0)
Not remember	10/308 (3.2%)	1.2 (0.4-3.8)	1.2 (0.3-4.2)

(1)-MDR= Multi drug resistance (resistance to at least Isoniazid and Rifampicin); (2)-CI= Confidence Interval; (3)-Adjusted for residence, gender, age and time to seek for treatment.

4.4. Molecular characteristics of *M.tuberculosis* population in Viet Nam

Molecular differentiation was performed on *M. tb* isolates by use of Spoligotyping and IS*6110*-RFLP.

4.4.1. Spoligotype patterns of *M.tuberculosis* strains from Viet Nam

A total of 580 strains isolated from new TB patients in Viet Nam during the study period and 100 historical strains that had been isolated in Southern part of Viet Nam from 1996 to 2008,

were analyzed by Spoligotyping. They were assigned to given *M. tb* families based on genotype results and by the algorithm of SPOTCLUST program using database SpolDB4 model (23).

Table 4.6: Spoligotype of *M. tb* strains isolated from 580 TB patients in Viet Nam, given families by lineage description of database SpolDB4

M.tb spoligotype	No of strains	Prevalence	Pooled family	M.tb family prevalence
Beijing	259	44.7%	Beijing	259 (44.7%)
EAI1	7	1.2%	EAI*	203 (35.0%)
EAI2	8	1.4%		
EAI4	94	16.2%		
EAI5	94	16.2%		
Family33	39	6.7%	Family33	39 (6.7%)
Family35	1	0.2%	Family35	1 (0.2%)
Family36	2	0.3%	Family36	2 (0.3%)
H37Rv	1	0.2%	H37Rv	1 (0.2%)
Haarlem1	3	0.5%	Haarlem	15 (2.6%)
Haarlem2	1	0.2%		
Haarlem3	11	1.9%		
LAM7	2	0.3%	LAM*	9 (1.6%)
LAM9	7	1.2%		
S	1	0.2%	S	1 (0.2%)
T1	35	6.0%	T	47 (8.1%)
T2	9	1.6%		
T3	2	0.3%		
X1	3	0.5%	X	3 (0.5%)
Total	580	100%		580 (100%)

*Abbreviations: EAI= East African Indian, LAM=Latin American Mediterranean (23)

Genotype of *M. tuberculosis* strains isolated during 2009-2010

As shown in Table 4.6, there is a big diversity in the *M.tuberculosis* population in which 19 distinct genotypes were recognized and divided into 11 families. The 2 dominating families were Beijing (44.7%) and East African-Indian (EAI) (35.0%). Other families present included Family33 (6.7%), Family35 (0.2%), Family36 (0.3%), H37Rv (0.2%), Haarlem (2.6%), Latin American and Mediterranean (LAM) (1.6%), S (0.2%), T (8.1%) and X (0.5%).

There was a significant difference of genotype patterns among the 3 regions (Figure 4.4) ($p<0.05$). We saw that the *M. tb* population from the North was more diverse, including 9 families and the Beijing family was the most prevalent (156/341, 45.7%) while our strains collection included 7 *M. tb* families among the isolates from the Middle and the South.

In addition, a significant difference of *M. tb* genotype distribution was found between the patients living in urban and rural areas ($p<0.05$). The *M. tb* population from urban areas was more diverse than that population found in patients living in rural areas of Viet Nam.

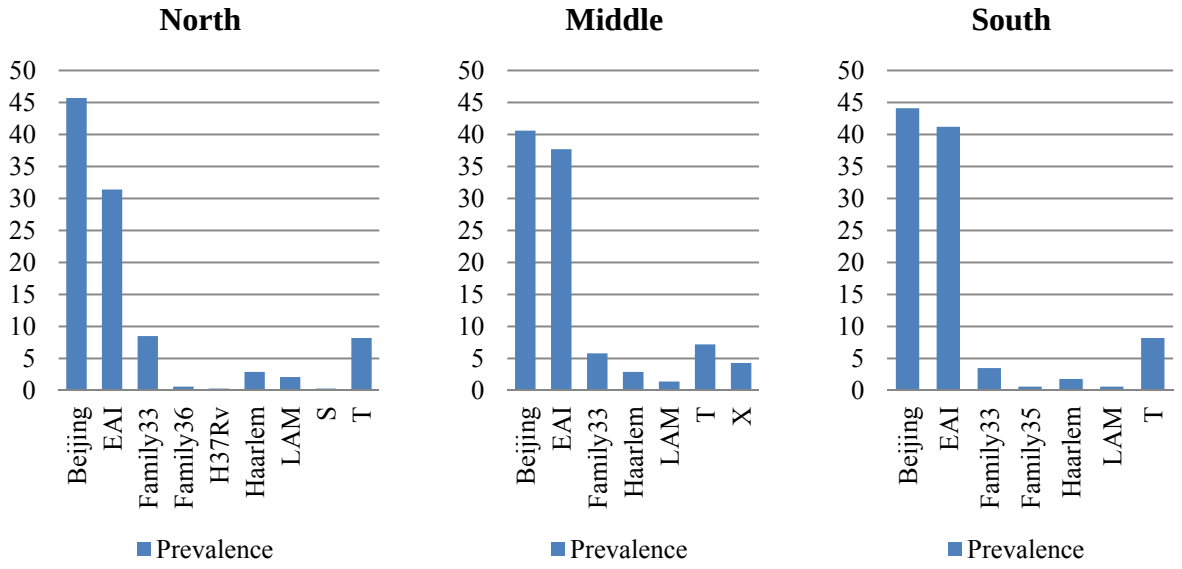


Figure 4.4: *M. tb* genotypes found in 3 different parts of Viet Nam. The North was more diverse in genotypes. The Beijing and EAI was most prevalent in all areas, although the Beijing strains were more often observed in the North, than in the Middle and South of Viet Nam. Abbreviations: EAI= East African Indian, LAM=Latin American Mediterranean (23).

There was less diversity among *M. tb* families among the HIV positive patients ($p<0.05$). Six lineages were found in the 40 co-infection patients. These included the Beijing (20/40, 50%), EAI (8/40, 20%), Family33 (5/40, 12.5%), H37Rv (1/40, 2.5%), Haarlem (2/40, 5%) and T (4/40, 10%) families.

A relatively low diversity was also found among the BCG vaccinated patients ($p<0.05$). Among these patients, there were six *M. tb* families included the Beijing (39/72, 54.2%), EAI (16/72, 22.2%), Family33 (7/72, 9.7%), Haarlem (1/72, 1.4%), T (7/72, 9.7%) and X family (2/72, 2.8%).

More *M. tb* genotypes were found among the younger age-groups ($p<0.05$). We observed 7/11 families in the group under 25 years, and 8/11 families in the 2 groups that included the ages of 25-34 and 35-44 years. We identified 6/11 families in the group that included ages 55-64, and 5/11 families in the 2 groups that included those 45-54 years old, and those patients above 65 years of age.

We also found an association between *M.tb* genotype infection and the delay time to seek medical diagnosis and treatment ($p<0.05$). Among the patients who came to hospitals within 3 months from first recognized symptoms, the Beijing strains were predominant (99/213 Beijing strains- 46.5%). Especially among 16 patients who came for treatment within 1 week of symptoms, the Beijing family was found in 8/16 (50%) patients, EAI in 6/16 (37.5%), Family33 in 1/16 (6.2%) and T in 1/16 (6.2%). In contrast, among those who did not seek treatment before the symptoms had persisted for 3 months or more, the EAI strains were more prevalent (43.8% EAI strains, 34.4% Beijing strains, 12.5% T strains and 9.3% other).

Out of 18 MDR-TB strains, 12 strains were assigned to Beijing family, 3 strains belonged to EAI family and the 3 families LAM, T, Family33 each had 1 MDR strains. Among Beijing strains, 79/217 (36.4%) strains were resistant to any of the first-line TB drugs while that of EAI strains were 23.5% (40/170) and other families were 34/89 (38.2%).

Genotype of historical *M. tuberculosis* strains isolated during 1996-2008

As described in Table 4.7, among 100 historical strains isolated from new TB patients during 1996-2008, Beijing family was the most prevalent with 58 strains, followed by EAI family (33 strains), then Haarlem family (4 strains), Family33 (3 strains) and T family (2 strains).

Table 4.7: Spoligotype of historical *M. tb* strains isolated in the South during 1996-2008 from 100 new TB patients, given families by lineage description of database SpolDB4.

***M.tb* spoligotype**	**No of strains**	**Pooled family**	***M.tb* family prevalence**
Beijing	58	Beijing	58%
EAI1	2	EAI*	33%
EAI2	5		
EAI4	13		
EAI5	13		
Family33	3	Family33	3%
Haarlem1	1	Haarlem	4%
Haarlem3	3		
T1	1	T	2%
T2	1		
Total	100		100%

*Abbreviations: EAI= East African Indian (23).

Table 4. 8: Full description of spoligotype patterns of 580 *M.tb* strains isolated from new TB patients in Viet Nam during 2009-2010 using lineage description of database SpolDB4 (23).

Spoligotyping Description	Spol genotype	Proba-bility	No of strains	Pooled Lineage	Preva-lence
□□■■■■■■■■	Beijing	0.99	234	**Beijing**	**259/580 (44.7%)**
□□□■■■■■■	Beijing	0.92	8		
□□■■□□□□□□□	Beijing	0.99	4		
□□■□■■■■■■■	Beijing	0.99	3		
□□□■■■■□■■■■	Beijing	0.99	3		
□□□■■■■■■■	Beijing	0.92	2		
□□□■■■■■■□■■	Beijing	0.99	2		
□□□■■■□■■■■■	Beijing	0.99	1		
□□□■■■■□□□□□	Beijing	0.99	1		
□□□■■■■■■■■□	Beijing	0.99	1		
■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□■■	EAI1	0.99	4	**EAI**	**203/580 (35.0%)**
■■□■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■□■■■	EAI2	0.99	3		
■■□■■■■■□□□□□□□□□□□□□□□□□■■■□□□□■□■■■■■■■■■	EAI2	0.96	2		
■■□■■■■□□□□□□□□□□□□□□□□□■■■□□□□■□■□□■■■■■■	EAI2	0.96	1		
■■■■■■■■■■■■□■■■■■□□□■■■■■■■□□□□■□■■■■■■■■■	EAI2	0.74	1		
■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.97	67		
■■■■■■■■■■■■□■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.98	8		
■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□□□■■■■■■■■■	EAI4	0.99	2		
□□■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■□□□□	EAI4	0.92	1		
■□□□□□□■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.77	1		
■□□■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.93	1		
■■■□□□□□□□□□□□□□□■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.51	1		
■■■□□□■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■□■■■	EAI4	0.92	1		
■■■□■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.98	1		
■■■■■□□□□□□□□□□□□□□□□■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.74	1		
■■■■■■□■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.99	1		
■■■■■■■□■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.98	1		
■■■■■■■■□■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.98	1		
■■■■■■■■■■□□□□□□■■■■■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.98	1		
■■■■■■■■■■■■■■■■■■■□■■■■□□■□□□□■□■■■■■■■■■	EAI4	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■□■■■■■	EAI4	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■□■■■■	EAI4	0.99	1		

■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■□□□□	EAI4	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■□□■■	EAI4	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■□■■■	EAI4	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■□	EAI4	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	EAI5	0.98	13		
■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	EAI5	0.97	11		
■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■■■	EAI5	0.95	5		
■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	EAI5	0.99	4		
■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□■■■■■■■	EAI5	0.98	4		
■■□■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□■■■■■■■	EAI5	0.99	3		
■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	EAI5	0.99	3		
■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	EAI5	0.98	2		
□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■■■	EAI5	0.95	1		
■□■■■■■■■■■■■■■■■■■■■■■■□■■□□□□■□■■■■■■■■■	EAI5	0.99	1		
■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	EAI5	0.99	1		
■■□■■■■■■■□■■■■■■■■■■■■■■■□□□□□□■■■■■■■■■	EAI5	0.98	1		
■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■□■■□■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□□□□□□■■■■■■■■□■■□□□□■□■■■■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□□□□□□■■■■■■■■■□■□□□□■□■■■■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□□■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□□■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	EAI5	0.98	1		
■■■■■■■■■■■□■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	EAI5	0.99	1		
■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□□□■■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■□■■■■■□□□□	EAI5	0.98	1		
■■■■■■■■■■■■■□□■■■■■■■■■■□■■□□□□■□■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■□□■■■□■■□□□□■□■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■□■■■■□■■□□□□■□■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	EAI5	0.98	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■□□□□■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□■■■■■■■■■	EAI5	0.97	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■■	EAI5	0.98	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■□□□■	EAI5	0.96	1		
□□□□□□■■■■■■■■■■□□□□□■□■■■■■■■■■■■■■■■■■■■■	Family33	1.00	1	**Family33**	**39/580 (6.7%)**
■■■□■■■■■■■■■■■■□□■■□□□□■■■■■■■■□□■■■■■■■■■■	Family33	0.99	1		
■■■□■■■■■■■■■■■■■■□□□□□□□□□□□□■□□■■■■■■■■■	Family33	0.99	1		

■■■■■■■■■■■■□■■■■■■■■■■■■□□□□□□■□□■■■■■■■■■■	Family33	0.99	1		
■■■■■■■■■■■■□■■■■■■■■■■■■□□■□□□■□■■■■■■■■■■	Family33	0.99	2		
■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■□■■■■■■■■■■	Family33	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■□■■□□□□■□■■■■■■■■■■	Family33	0.99	20		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■	Family33	0.99	6		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■	Family33	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■	Family33	0.99	1		
■■	Family33	1.00	4		
■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Family35	0.98	1	**Family35**	**1/580 (0.2%)**
□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	Family36	0.99	1	**Family36**	**2/580 (0.3%)**
□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■■■■■■■■■■■	Family36	1.00	1		
■■■■■■■■■■■■■■■■■□□□□■■■■■■■■■■□□□□■■■□■■■	H37Rv	0.96	1	**H37Rv**	**1/580 (0.2%)**
■■■■■■■■■■■■■■■□□■■■■■■■■□□□□□□■□□□□■■■□■■■	Haarlem1	0.99	1	**Haarlem**	**15/580 (2.6%)**
■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■□□□□■■■■■■■	Haarlem1	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■	Haarlem1	0.97	1		
□□□□□□□□□□□□□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	Haarlem2	0.99	1		
■■■■■■□■■■■■□■■■■■■■■■■■■■■■□■□□□□■■■■■■■	Haarlem3	0.77	2		
■■■■■■■■■■■■■■■■■□■■■■■■□■■□□□□□□□□■■■■■■■	Haarlem3	0.70	1		
■■■■■■■■■■■■■■■■■■■■■□□□■■■■□■□□□□■■■■■■■	Haarlem3	0.72	1		
■■■■■■■■■■■■■■■■■■■■■■□□■■■■■□■□□□□■■■■■■■	Haarlem3	0.72	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□■■■	Haarlem3	0.72	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	Haarlem3	0.77	5		
■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■	LAM7	0.98	1	**LAM**	**9/580 (1.6%)**
■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□■□□□□■■■■■■■	LAM7	0.99	1		
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■□■■	LAM9	0.99	1		
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	LAM9	0.99	6		
■■■■■■■□□□□■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	S	0.76	1	**S**	**1/580 (0.2%)**
■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	0.99	2	**T**	**47/580 (8.1%)**
■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	0.99	2		
■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■□□□□■■■■■■■	T1	0.99	1		
■■■■■■■■■■■■■□■■■■□□■■■■■■■■■■■■□□□□■■■■□■■	T1	0.99	2		
■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	0.99	2		
■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	T1	0.99	3		

■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■■■■	T1	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□□□■■■■■■	T1	0.99	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□□■■■■	T1	0.99	2		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□■■■■	T1	0.99	2		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T1	0.99	4		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	T1	0.99	14		
■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■■□□□□	T2	0.98	1		
■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□□□□■■■□□□□	T2	0.97	4		
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□□□	T2	0.97	5		
■■■■■■■■□□□□□□□□□□□□■■■□□□□□□□□□■□□□□■■■■■■■	T3	0.99	1		
■■■■■■■■■■■■■■■■□□□■■■■■■■■■■■■■□□□□■■■□■■■	X1	0.61	1		
■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■□■■■	X1	0.59	1	**X**	**3/580 (0.5%)**
■■■■■■■■■■■■■■■■■□■■■■■■■■■■□□□□■□■■■■■■■■■	X1	0.59	1		

4.4.2. Beijing genotype prevalence in Viet Nam

Beijing was the most prevalent *M. tb* family in the current collection from Viet Nam. It was found more in the Northern part of Viet Nam with 156/341 (45.7%) strains. While the Southern and Middle parts had 75/170 (44.1%) and 28/69 (40.6%) Beijing strains respectively. This difference was not statistically significant ($p>0.05$).

Demographic factors associated with Beijing genotype infection is presented in table 4.9. Patients living in urban areas had 1.6 (95%CI: 1.1-2.4) times greater possibility of being infected with the Beijing strains than patients living in rural areas.

We observed a significant difference of Beijing genotype prevalence in the genders ($p<0.05$). There was a 1.7 (95%CI: 1.1-2.6) times higher risk for women to be infected by the Beijing genotype than men.

Additionally, there was a minor negative correlation between age and the risk of being infected with the Beijing genotype and it is significantly different from zero ($p<0.05$). Beijing strains were associated with younger age. This was especially evident in the youngest group of less than 25 years of age, which had 3.3 (95%CI: 1.5-7.0) times higher risk of Beijing infection than the oldest group of more than 65 years.

We also observed a strong correlation between Beijing genotype and drug resistance. Patients who were diagnosed with MDR-TB or resistance to any of the given anti-TB drugs had a higher possibility of being infected with the Beijing genotype ($p<0.05$) (Table 4.10).

Table 4.9: The association of Beijing genotype and demographic factors in Viet Nam in univariable and multivariable analysis

		Beijing genotype		Crude OR			Adjusted OR		
	No	**No**	**%**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Year									
2009	275	124	47.9%	1.0	0.7-1.4	0.841	1.0	0.6-1.5	0.906
2010	305	135	52.1%	1			1		
Region									
North	341	156	45.7%	1.1	0.7-1.5	0.722	1.6	1.0-2.6	0.055
Middle	69	28	40.6%	0.9	0.5-1.5		0.9	0.5-1.7	
South	170	75	44.1%	1			1		
Residence									
Rural	259	103	39.8%	1		**0.028**	1		**0.018**
Urban	317	155	48.9%	1.4	1.0-2.0		1.6	1.1-2.4	
Gender									
Male	438	182	41.6%	1		**0.003**	1		**0.012**
Female	138	77	55.8%	1.8	1.2-2.6		1.7	1.1-2.6	
Age group									
<25	67	40	59.7%	3.6	1.7-7.5	**0.007**	3.3	1.5-7.0	**0.017**
25-34	132	62	47.0%	2.2	1.1-4.1		2.1	1.1-4.1	
35-44	136	66	48.5%	2.3	1.2-4.4		2.4	1.2-4.7	
45-54	113	47	41.6%	1.7	0.9-3.4		1.7	0.8-3.3	
55-64	61	22	36.1%	1.4	0.6-2.9		1.4	0.6-3.0	
65+	62	18	29.0%	1			1		
DST									
Sensitive	308	126	40.9%	1		0.017			
DR-any [1]	153	79	51.6%	1.5	1.0-2.3				
MDR[2]	18	12	66.7%	2.9	1.0-7.9				

OR: odd ratio; CI: confidence interval; Adjusted for year of collection, region, living area, gender and age group. (1)-Resistance to any first-line drugs, including Isoniazid, Rifampicin, Streptomycin and Ethambutol. (2)-Multi drug resistance (resistance to at least Isoniazid, Rifampicin)

Moreover, we found an association of Beijing genotype and *M. bovis* BCG vaccination history in the Southern part of Viet Nam after further analysis by region ($p<0.05$). The Beijing genotype was the most prevalent genotype among *M. bovis* BCG vaccinated patients in our material. This group included 14/21 (66.7%) patients. Only 3/12 (25%) patients were infected with the Beijing genotype among the unvaccinated group. This difference was not significant for the Northern and Middle parts of Viet Nam (Table 4.11).

Table 4.11: The Beijing genotype prevalence among TB patients with different BCG-vaccination history in 3 regions of Viet Nam.

	BCG History		
	Vaccinated	Unvaccinated	Uncertain
North	13/23 (56.5%)	17/41 (41.5%)	126/277 (45.5%)
Middle	12/28 (42.9%)	6/14 (42.9%)	10/27 (37.0%)
South	14/21 (66.7%)	3/12 (25.0%)	58/137 (42.3%)

When comparing the 580 samples received during the study period to 100 historical samples isolated during 1996-2008, we found a big difference in the prevalence of the Beijing genotype (Table 4.12). This difference was statistically significant ($p<0.05$). A total of 58% of the *M. tb* stains isolated from 1996 -2008 were assigned to the Beijing family and 44.7% of strains collected during study period from 2009 to 2010 were assigned to this family.

Table 4.12: The Beijing genotype prevalence in recently collected and historical strains

	Beijing Genotype		**non-Beijing Genotype**	
	No	**%**	**No**	**%**
Isolated 2009-2010	259/580	44.7%	321/580	55.3%
Isolated 1996-2008	58/100	58%	42/100	42%
Total	317/680	46.6%	363/680	53.4%

4.4.3. DNA fingerprinting IS*6110*-RFLP patterns of Beijing strains in Viet Nam

Based on results obtained by Spoligotyping method, the Beijing genotype strains were selected for further differentiation by the use of IS*6110*-RFLP method. A total of 205/259 Beijing strains collected during 2009-2010 and 47/58 strains isolated during 1996-2008 were analyzed. Among those, 113/252 (44.8%) strains were from the North, 19 (7.5%) strains from the Middle and 120 (47.7%) strains from the South. We were unable to include 54/209 and 11/58 strains that were lost to contamination (Figure 4.5). The IS*6110*-RFLP pattern of 252 Beijing strains from the study population is presented in Figure 4.6.

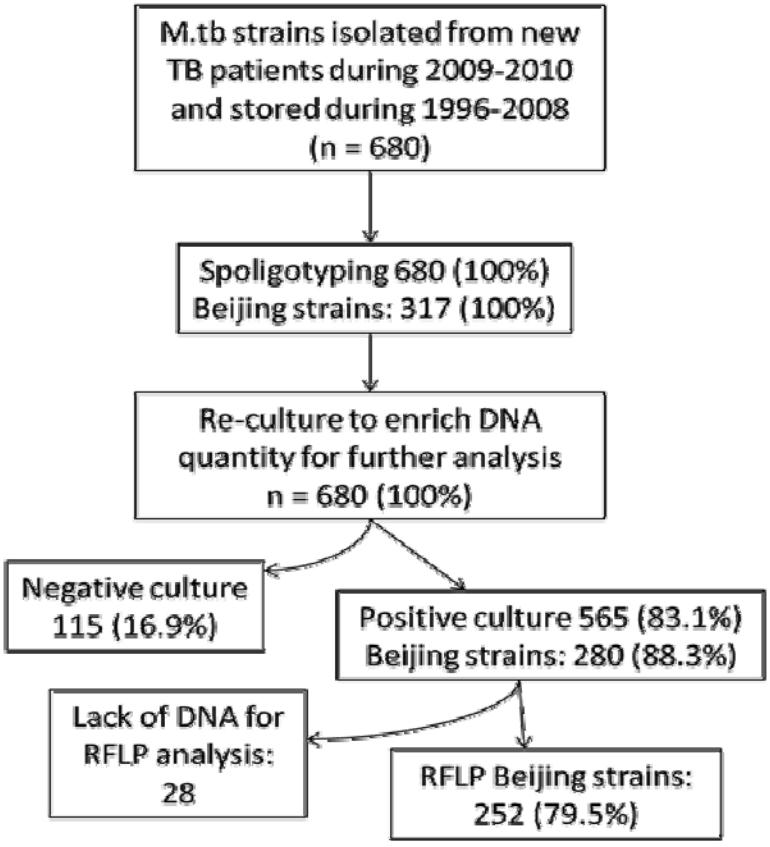


Figure 4.5: Enrollment of strains in molecular methods. 680 strains were analyzed by Spoligotyping method and 252 strains were further analyzed by IS*6110*-RFLP method.

Figure 4.6-1: IS*6110*-RFLP pattern dendogram of *M. tb* isolates assigned to the Beijing family (n = 252). The banding patterns (right panel) were ordered by similarity (left panel).

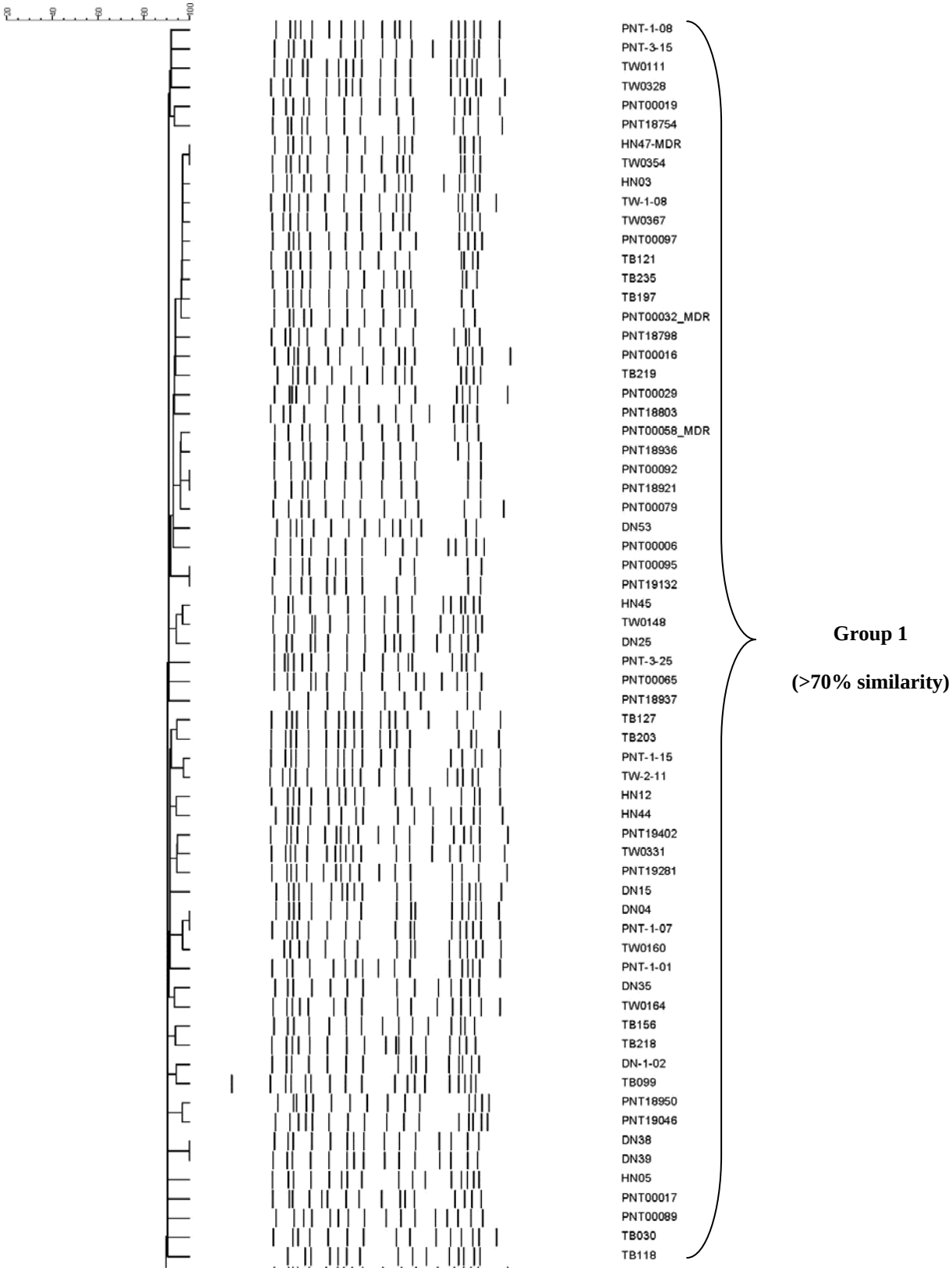


Figure 4.6-2: RFLP pattern dendogram of *M.tb* Beijing strains (n = 252). The banding patterns (right panel) were ordered by similarity (left panel) (continued).

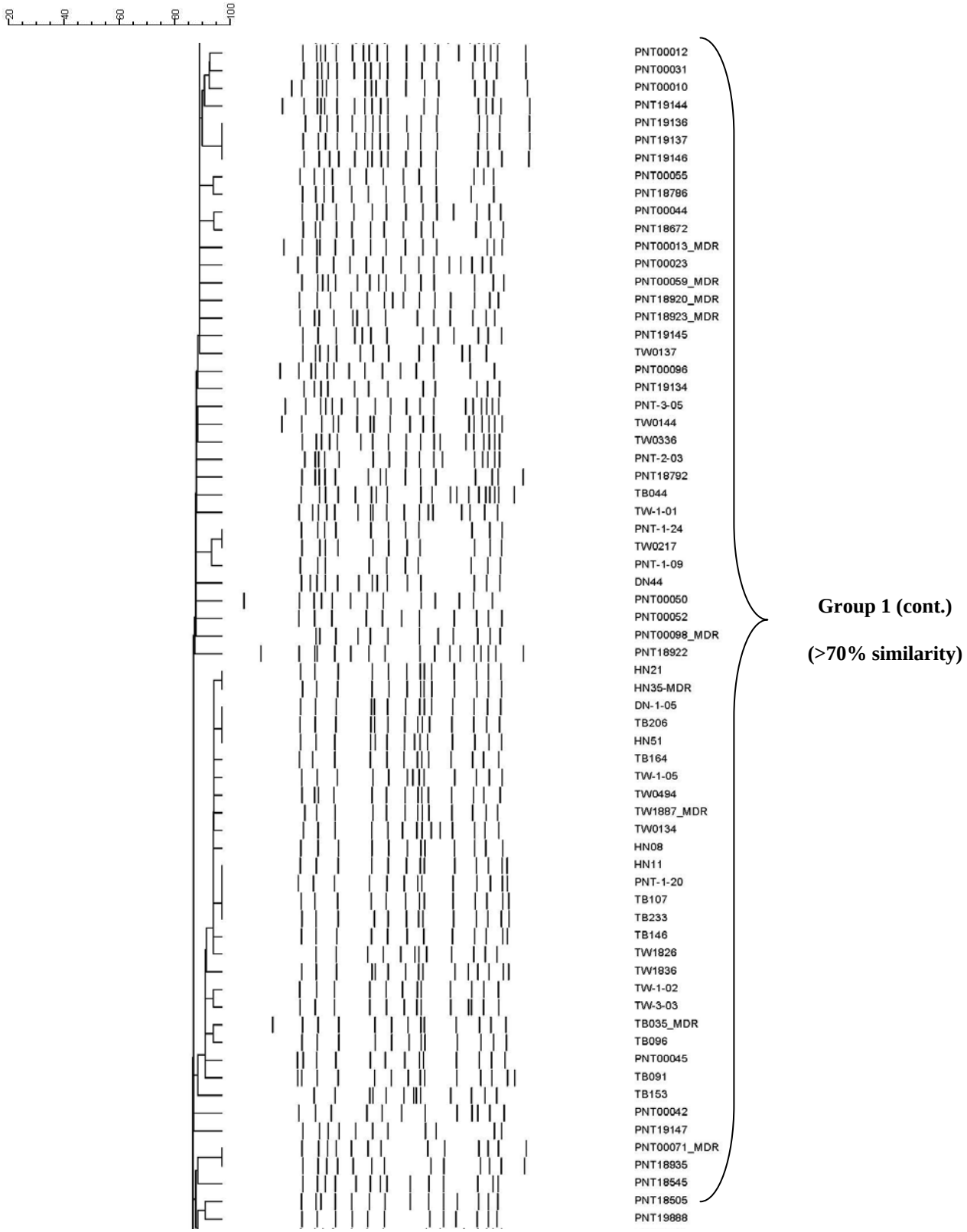


Figure 4.6-3: RFLP pattern dendogram of *M.tb* Beijing strains (n = 252). The banding patterns (right panel) were ordered by similarity (left panel) (continued).

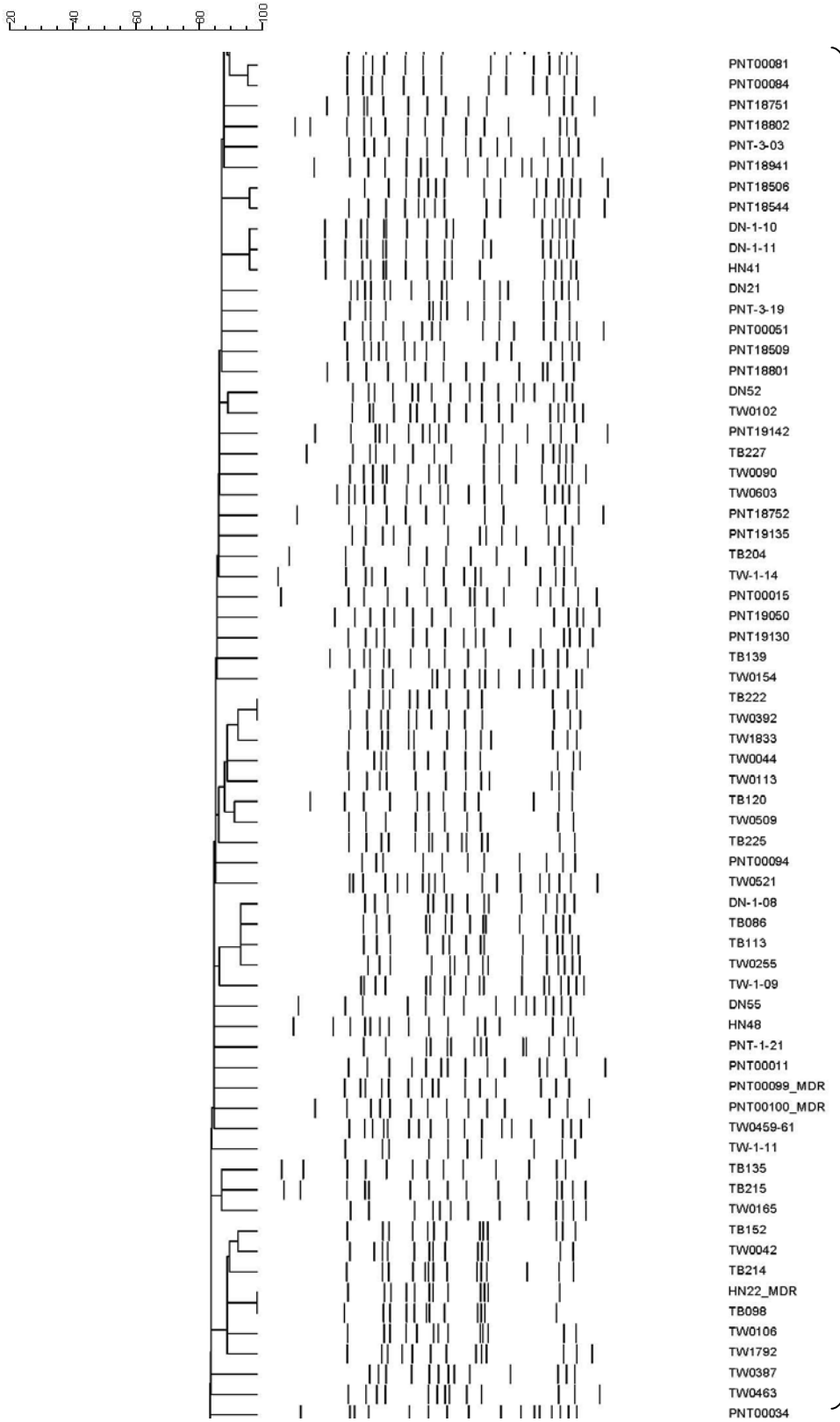
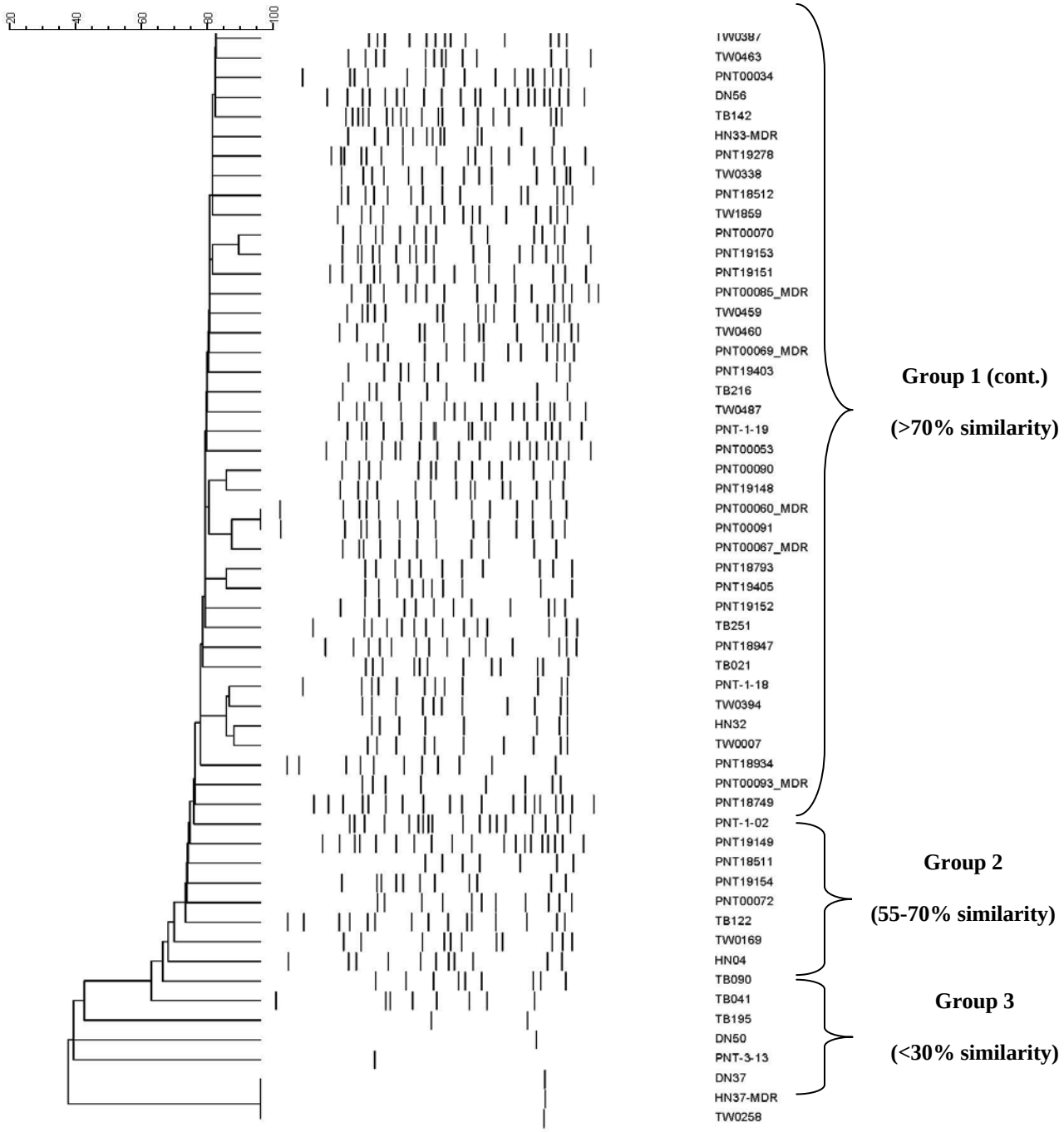


Figure 4.6-4: RFLP pattern dendogram of *M.tb* Beijing strains (n = 252). The banding patterns (right panel) were ordered by similarity (left panel) (continued).



The detailed characteristics of the IS*6110*-RFLP patterns revealed a large diversity among the *M. tb* Beijing strain population in the current collection from Viet Nam. Three distinct groups were identified, including a main group to which 238/252 strains were assigned. This group was highly homogenous (more than 70% of similarity in RFLP patterns) although including a few identical (clustered) strains. Interestingly, the 47 historical strains isolated during 1996-2007 were distributed evenly in this group. The second group included 8/252 strains, which shared at least 55% similarity. All of these were heterogeneous carrying unique IS*6110*-RFLP patterns. The last group that included 6/252 strains carried less than 3 copies of IS*6110* thus representing "low IS*6110*-copy strains", possibly members of the "atypical Beijing" or "old Beijing" strains.

Comparing the IS*6110*-RFLP patterns of MDR-TB Beijing strains, 7/22 were identical to non-MDR-TB strains. Also, 4/22 MDR-TB strains were highly homogenous and 11/22 were carried unique IS*6110*-RFLP patterns.

Moreover, we observed the differences between IS*6110*-RFLP patterns of *M. tb* strain population from the North and the South of Viet Nam which are illustrated in Figure 4.7 and 4.8. The Middle region included only 19 strains therefore were not analyzed further. The results shown that *M. tb* strain population from the North was more heterogeneous, distributed in several subgroups, while the Southern strain population was homogeneous that most of the strain dispersed into one large group.

Figure 4.7: IS*6110*-RFLP pattern dendrogram of *M. tb* Beijing strains from the North of Viet Nam (n=113). The banding patterns (right panel) were ordered by similarity (left panel).

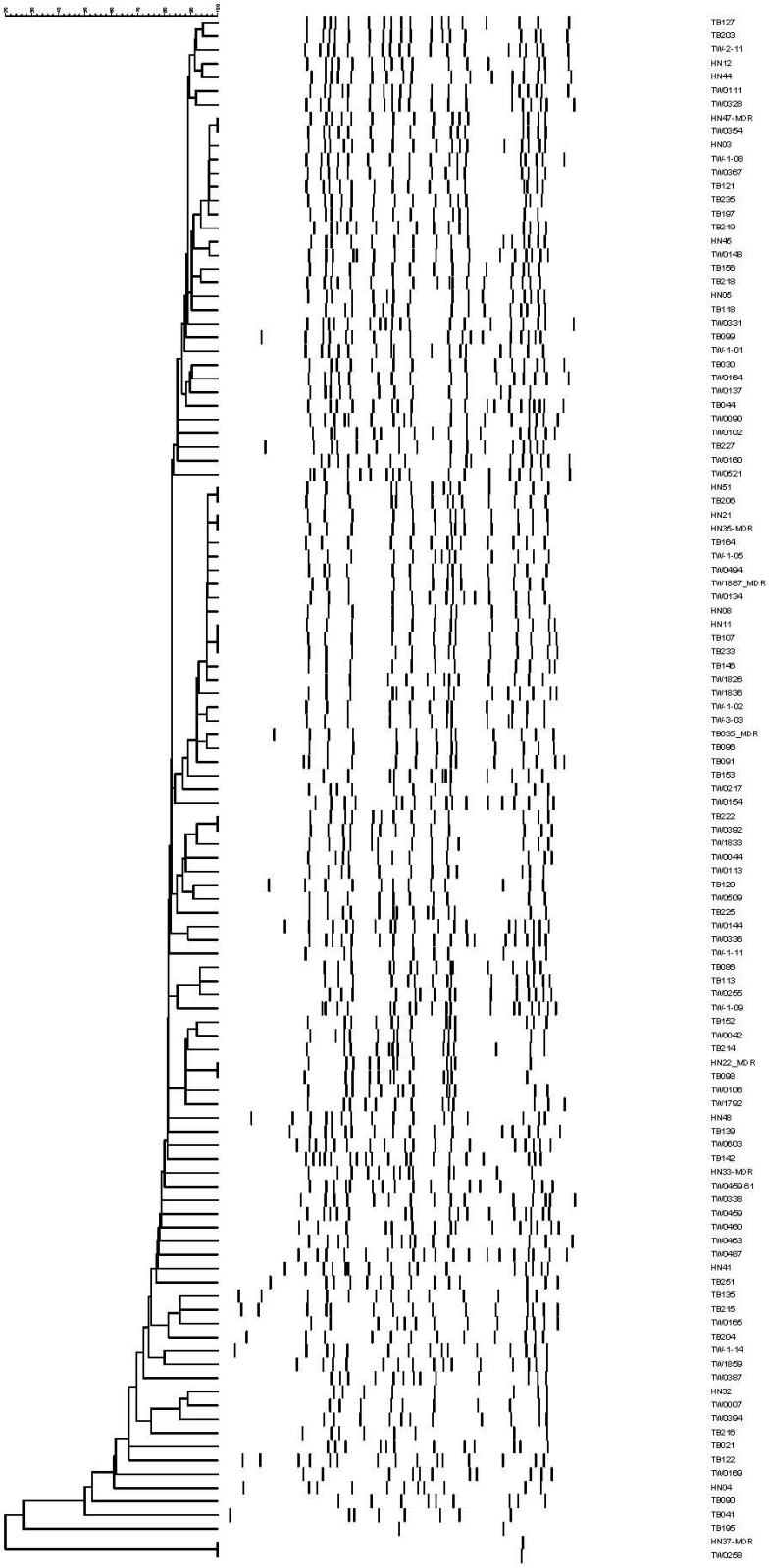
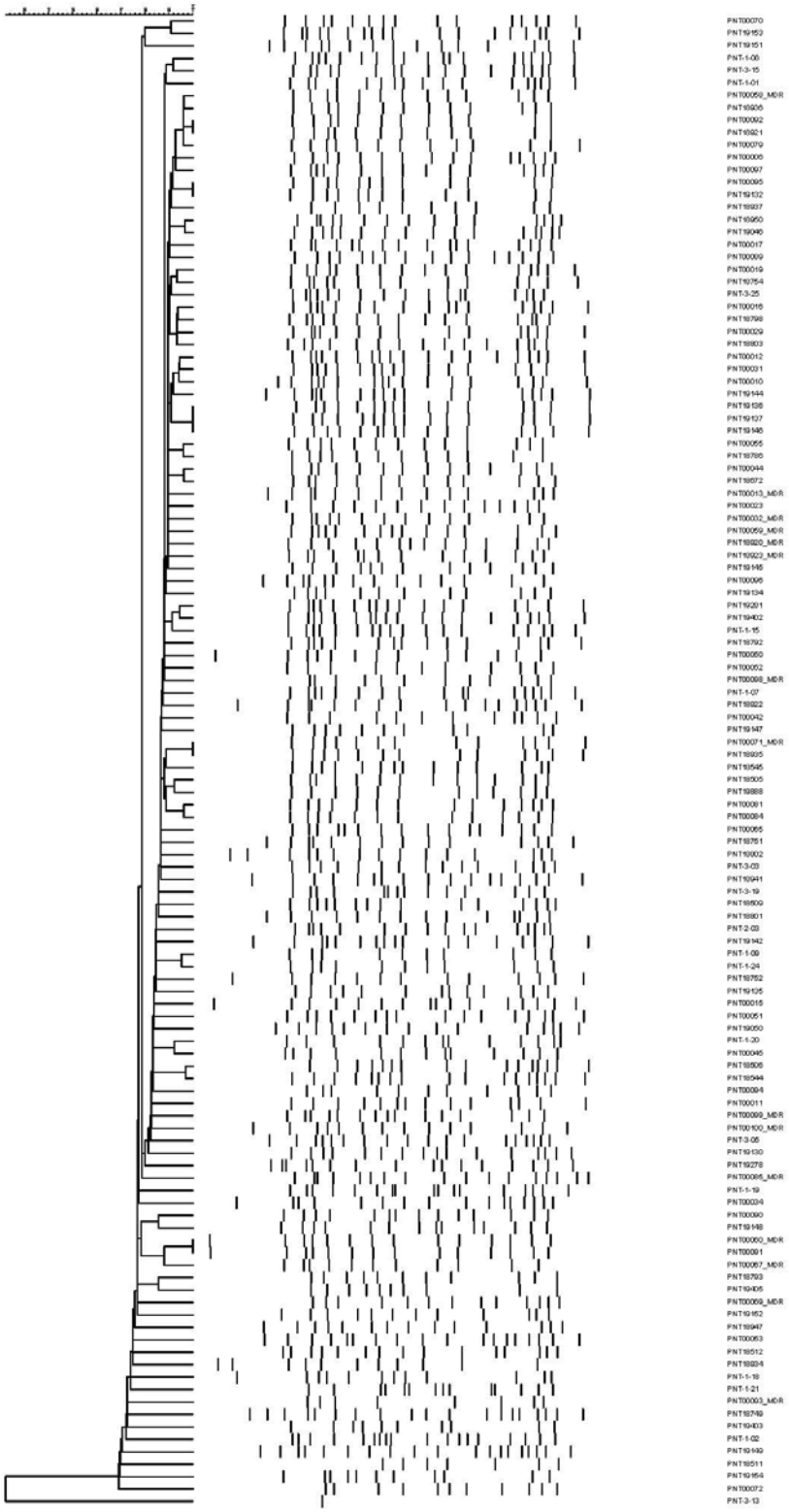


Figure 4.8: IS*6110*-RFLP pattern dendrogram of *M. tb* Beijing strains from the South of Viet Nam (n=120). The banding patterns (right panel) were ordered by similarity (left panel).



CHAPTER V: DISCUSSION

This is the most up-to-date molecular epidemiological study with the largest number of samples distributed across the three different regions of Viet Nam that intended to investigate the *M. tb* population structure and so to better understand the TB epidemic in Viet Nam.

As mentioned in the methodology part, the study was based on a cross-sectional design and convenient sampling method which are less costly and less time-consuming. It has increased the sample collecting probability and made us able to exceed the calculated minimal sample size within a limited amount of time and funding. Still there remains some possibility of bias and we cannot say it is fully representative of the whole country. In order to relatively secure the representativeness of *M. tb* population in Viet Nam, we have recruited participants from the NTP and five TB local hospitals in the three main regions of Viet Nam, the North, the Middle and the South. The sample recruitment took places in all the TB centers and units located in urban and rural areas that these hospitals are responsible for.

The study population was recruited from all newly diagnosed patients admitted to the five TB hospitals that have underwent chest X-ray examination, sputum smear microscopy, *M. tb* culture positive result and voluntarily agreed to participate in the study. Our study has limitations for those who have chosen treatment at private sectors, as well as false culture-negative and extra-pulmonary TB patients. Small children are also difficult-to-reach population.

With the availability of modern techniques, the ideal method to access genetic variety of *M. tb* strains is the comparison of the whole genome sequences. However, it requires bioinformatics knowledge, time, and extensive funding. Therefore, to differentiate among *M. tb* strains, the method of comparing fractions of genome to obtain genotypes is more relevant and less time-consuming (53). Among the most commonly used genotyping methods, Spoligotyping is the simplest technique and IS*6110*-RFLP is considered as a gold standard for *M. tb* strain genotyping. In addition, the IS*6110*-RFLP method is more powerful in discriminating strains that have more than 6 copies of IS*6110* insertion while Spoligotyping method has been useful in differentiating among *M. tb* isolates with low-copy-number of IS*6110* insertion (25). When used Spoligotyping alone, other studies (31;33) has shown the limitation in discriminatory power because it targets a single locus that accounts for less than 0.1% of the *M. tb* genome, unlike

IS6110-RFLP analysis, which examines the distribution of IS*6110* throughout the entire genome (25). In another study which used IS*6110*-RFLP alone, 39% of the examined strains had less than 6 copies of IS*6110* insertions in *M. tb* genome and 1.8% had no IS*6110* (33). Therefore, recognizing the importance of using both techniques for more accuracy and discriminatory genotyping results, although it is more expensive and time-consuming, we have practiced both methods in our study selectively. After identification of *M. tb* genotype by Spoligotyping, all Beijing genotype strains were analyzed further by IS*6110*-RFLP.

During the entire study, a total of 580 new TB patients were recruited. The majority of the study population was male (3 times the number of female) which resemble the same gender distribution of all new TB cases in the nation. More patients were recruited from the North which mostly lived in rural areas, whereas patients from the Middle and South mostly lived in urban areas. But in general, the numbers of rural patients were relatively equivalent to that of urban patients. The female patients were comparatively younger than the male patients.

The majority of patients (335/580- 57.8%) from the study did not remember since when they started having symptoms indicative of TB. Among those who reported symptoms, most of them were present in hospitals within 3 months (6.5% within one week, 35.5% in one month and 45% in 3 months). The remaining 13% patients had sought the treatment after 3 months to more than one year of symptom-debut. The large number of patients that did not remember their symptom duration was assumed to be mainly presenting the long delay time. The MDR-TB was associated with delay time to seek for treatment. The risk of having MDR-TB was much higher in patients who came late to the hospitals after 3 months of experiencing symptoms.

As described in the Result part, our 580 *M. tb* strains isolated from new TB patients during 2009-2010, were assigned to 19 genotype families and 11 pooled families, based on Spoligotyping results and the algorithm of SPOTCLUST program using database SpolDB4 model. Among those, the two families, Beijing and EAI were the most prevalent throughout all 3 regions of Viet Nam. Other families were found in small proportions. The distribution of *M. tb* strains in our study was matched with the general picture of East Asia (23).

The main finding in this study is that we have found a strong evidence of declining in Beijing genotype prevalence among the circulating strains in Viet Nam. From Spoligotyping results of 100 historical strains isolated during 1996-2008 in our study, the Beijing genotype was

found predominantly in 58% of strains. Although the result might be biased due to the small numbers of historical strains and all originated from the South, it is similar to a study conducted in 1998-1999. This is the first study targeted to the Beijing genotype epidemic in Viet Nam and found the wide spread of this genotype at the rate of 54% (301/563) prevalence (34). In our new collection of strains, the prevalence was decreased largely to 44.7%. Additionally, another study carried out in Southern rural setting during 2003-2005 had established a 33.2% prevalence of Beijing strains among new TB cases (32). While in our study with the same setting, 21.4% of strains were identified as Beijing genotype which is a significant decline of Beijing strain epidemic. This proportion is comparatively lower than that of other countries in East Asia such as China 81.9% (86/105) (54), Hong Kong 68.5% (243/355) (51), Thailand 57.1% (84/147) (52) and South Korea 78% (75/96) (53). This decline may due to the fact that other family is expanding and taking dominant control, i.e. EAI family. We have found the EAI genotype in 33% of Southern historical strains and increased to 41% of newly collected Southern strains. However, this finding needs more retrospective analysis on historical strains of both the North and Middle to confirm. Other possible explanation for the reduction of the Beijing strain prevalence is the enhancement of nationwide TB control program that recently catch more rural TB patients than earlier, while Beijing genotype is more prevalent in urban than in rural.

Among the BCG vaccinated patients, 6/11 families were found including Beijing, EAI, Family33, Haarlem, T and X family. The Beijing genotype was predominant in 54.2% of strains. Also, among the TB patients who came to hospitals within 3 months from first recognized symptoms, the Beijing strains were the most prevalent (99/213 Beijing strains- 46.5%). Especially among 16 patients who came for treatment within 1 week of symptoms, the Beijing family was found in 8/16 (50%) patients, EAI in 6/16 (37.5%), Family33 in 1/16 (6.2%) and T in 1/16 (6.2%). In contrast, among those who did not seek treatment before the symptoms had persisted for 3 months or more, the EAI strains were more prevalent (43.8% EAI strains, 34.4% Beijing strains, 12.5% T strains and 9.3% other). This could indicate that the Beijing strains might cause more severe or easy-to-recognize symptoms that encourage the patients seek medical help earlier than with other *M. tb* infections.

In addition, Beijing genotype in Viet Nam was associated with patients' residence, gender, age group, drug resistance but not with BCG vaccination history. Living in urban areas, being female and of younger age are risk factors for being infected with Beijing genotype. Also

MDR and/or resistance to any of the first-line drugs are more likely as a result of Beijing strain. The results from the previous studies show similar findings in Viet Nam (31-33).

Moreover, the detailed characteristics of the IS*6110*-RFLP patterns revealed a large diversity among the *M. tb* Beijing strain population in the current collection from Viet Nam. Three main groups were identified, including a main group to which 238/252 strains were assigned. This group was highly homogenous (more than 70% of similarity in RFLP patterns) although with few identical (clustered) strains. This may be indicative of an established epidemic as most strains had similar, but not identical isolates included. Interestingly, the 47 strains isolated during 1996-2008 were distributed evenly in this group, suggesting that this possible evolution had been ongoing in Viet Nam since at least 1996. When comparing IS*6110*-RFLP patterns of MDR-TB Beijing strains, 7/22 were identical to non-MDR-TB strains indicating that MDR-TB had been acquired after transmission. It suggests that the follow-up of the patients during and after treatment need to be enhanced in order to reduce the development of drug resistance.

The differences between the IS*6110*-RFLP strain patterns from the North and the South are shown in Figure 4.7 and 4.8. Most Southern *M. tb* strains were distributed in one large group indicating strain dissemination. Whereas, Northern strains were assigned into different subgroups indicating the diversity. In general, it suggests that *M. tb* populations of 2 regions were possibly derived from different ancestor strains that distinguished with each other by both the number of IS*6110* insertion and their transposition sites throughout the genome. This picture was also seen in 1994-1996 study (33). Additionally, the atypical Beijing ("old Beijing") patterns with low-copy of IS*6110* is tended to be eliminated from the population. In previous study, the atypical Beijing genotype was found in 63/168 (37.5%) strains, while it was only 6/252 (2.4%) strains in our study. Further analysis on changes in *M. tb* populations of both regions needs to be performed to evaluate the change in TB epidemic of Viet Nam.

CHAPTER VI: CONCLUSION

Mycobacterium tuberculosis (*M. tb*) population in Viet Nam was comprised of 19 different genotype families that assigned to 11 pooled families by Spoligotyping results. Among those, the two families, Beijing and East-African-Indian (EAI) were the most prevalent throughout all 3 regions of Viet Nam. Other families were found in small proportions.

The Beijing genotype has been predominating in the nation since 1996. However, its prevalence is declining and was at the rate of 44.7% in 2009-2010, while EAI family seems to be expanding and taking dominant control.

In Viet Nam, Beijing genotype is associated with patients' residence, gender, age group, drug resistance but not with BCG vaccination history. Living in urban areas, being female, of younger age and having MDR and/or resistance to any of the first-line drugs are risk factors for being infected with Beijing strains.

The detailed characteristics of the IS*6110*-RFLP patterns revealed a large diversity among the *M. tb* Beijing strain population. Three main groups were identified by the similarity of IS*6110*-RFLP patterns, in which most of the Beijing strains were belonged to one group indicating an established epidemic as most strains were similar, but not identical isolates included. This possible evolution had been ongoing in Viet Nam since at least 1996.

One third of the MDR-TB strains were identical to non-MDR-TB strains demonstrating that MDR-TB had been acquired after transmission. Thus, the follow-up of the patients during and after treatment need to be enhanced.

The differences between the IS*6110*-RFLP strain patterns of the North and the South suggested that the *M. tb* Beijing populations of the two regions were possibly derived from different ancestor strains. The Northern strains were diverse, whereas the Southern strains were disseminated. The atypical Beijing ("old Beijing") patterns with low-copy of IS*6110* is tended to be eliminated from the *M. tb* population of both regions.

Further research on dynamic of Viet Nam *M. tb* population needs to be performed in order to evaluate the change in TB epidemic of Viet Nam.

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ANNEX 1: MOLECULAR METHOD – SPOLIGOTYPING PROTOCOL

A. In vitro amplification of spacer DNA by PCR

Principle

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal Mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Löwenstein) can also serve as template. The PCR products are labeled with biotin, because primer DRa is biotinylated.

Procedure

1. Always include chromosomal DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG P3 as positive controls. Use water as a negative control.
2. Prepare the reaction mixture:

   2 µl template DNA

   3 µl primer DRa (0.2 µmol/µl)

   3 µl primer DRb (0.2 µmol/µl)

   20 µl 2×TaqPCR MasterMix

   12 µl MQ water (to a final volume of 40 µl)

3. Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling:

   3 min 94 °C 1 Cycle

   1 min 94 °C

   1 min 55 °C

   30 sec 72 °C 25 Cycles

   7 min 72 °C 1 Cycle

   ∞ 4 °C

B. Hybridization with PCR product and detection

Hybridization of the biotin-labeled PCR products to the immobillized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

1. All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):

   2×SSPE/0.1 % SDS, 42 °C,

   2×SSPE/0.5 % SDS, 60 °C,

   2×SSPE/0.5 % SDS, 42 °C.

   2×SSPE, room temperature.

2. Add 25 µl of the PCR products to 150 µl 2×SSPE/0.1 % SDS.
3. Heat-denature the diluted PCR product for 10 min at 100 °C and cool on ice immediately.
4. Wash the membrane for 5 min at 42 °C in 250 ml 2×SSPE/0.1 % SDS.
5. Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.
6. Remove residual fluid from the slots of the miniblotter by aspiration.
7. Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60 °C on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots.
8. Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.
9. Wash the membrane twice in 250 ml 2×SSPE/0.5 % SDS for 5 min at 60 °C.
10. Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.
11. Add 5 µl streptavidin-peroxidase conjugate (500 U/ml) to 14 ml of 2×SSPE/0.5 % SDS, and incubate the membrane in this solution for 60 min at 4 °C in the rolling bottle.
12. Wash the membrane twice in 250 ml of 2×SSPE/0.5 % SDS for 10 min at 42 °C.
13. Rinse the membrane twice with 250 ml of 2×SSPE for 5 min at room temperature.

14. For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 16 ml ECL detection liquid.
15. Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 1 min.
16. If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

C. Regeneration of the membrane

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times.

1. Wash the membrane twice by incubation in 1 % SDS at 80 °C for 30 min.
2. Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.
3. Store the membrane at 4 °C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

ANNEX 2: MOLECULAR METHOD – IS*6110*-RFLP PROTOCOL

A. Preparation of labelled IS*6110* probe

Amplification of IS*6110* sequence

Primer (Invitrogen):

Fw: CGTGAGGGCATCGAGGTGGC

Rv: GACGTAGGCGTCGGTGACAAA

PCR mix:

25 µL Taq PCR master mix 2X (Affymetrix USB, USA)

1 µL forward primer 50 pM

1 µL reverse primer 50 pM

2 µL template DNA (M. Bovis)

21 µL H2O

PCR cycles: 94^0C – 5 min

94^0C – 1 min
60^0C – 1 min
72^0C – 1 min
} 30 cycles

72^0C – 10 min

Purification of PCR product: using kit illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK).

Determine DNA concentration by using Spectrophotometer NanoDrop 1000 (Thermo Scientific, USA)

Labeling Probe

Amersham ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare, UK)

1. Dilute the DNA to be labeled to a concentration of 10 ng/µl using the water supplied

2. Denature 100 ng of the DNA sample (10 µl) by heating for 5 min in a boiling water
3. Immediately cool the DNA on ice for 5 min. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube
4. Add an equivalent volume of DNA labeling reagent (10 µl) to the cooled DNA. Mix gently but thoroughly.
5. Add the glutaraldehyde solution, use a volume equivalent to the volume of the labeling reagent (10 µl). Mix thoroughly. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube
6. Incubate for 20 min at 37^0C (labelling probe < 300bp)
7. If not used immediately, the probe can be held on ice for a short period, for example 10-15 min
8. Labelled probes may be stored in 30% glycerol at -15 to -30^0C for up to six months

B. Genomic DNA extraction from *M. tb*

- Kill the bacteria by heating for 1 hour at 80oC, and cool at RT
- Add 20 µl lysozyme (10mg/ml) and incubate 37oC overnight, mix gently
- Add 5 µl proteinase K (10 mg/ml) – mix lightly
- Add 70 µl of 10% SDS, mix them and vortex, and incubate for 10 – 15 minutes at 65oC
- Add 100 µl of 5M NaCl and 100 µl of CTAB/NaCl solution pre-warmed at 65oC, and vortex until the liquid content becomes milky (or mix them 10 – 20 seconds). Incubate for 10 minutes at 65oC
- Add 750 µl of Chloroform/isoamyl alcohol (24:1) and vortex for at least 10 seconds
- Centrifuge for at least 30 minutes at 13,000 rpm
- Transfer the aqueous phase (upper layer) to a fresh micro centrifuge tube, by pipetting small aliquots of e.g. 180 µl
- Add 450 µl (0.6 volume) of isopropanol – mix well

- Manually move the tube slowly upside down to precipitate the nucleic acids and estimate the amount of 1X TE in which the DNA should be redissolved later. write the estimated volume e.g. on the tube
- Place at -20oC for at least 30 minutes (± over night)
- Take out from Freezer, place it in RT
- Centrifuge in 30 minutes at 13,000 rpm
- Discard most of supernatant, leave about 20 µl (3mm height) above the pellet
- Add 900 µl of cold 70% Ethanol (from the -20^{o}C freezer) and turn the tube a few time upside down to wash the DNA precipitate.
- Centrifuge in 10 minutes at 13,000 rpm
- Discard most of the supernatant; leave about 20 µl (3 mm height) above the pellet
- Dry at RT
- Add 20 - 30 µl of TE buffer (1X TE) to dissolve DNA - vortex
- Place in freezer – 20^{o}C

Measurement of DNA concentration

For further analysis, it is necessary to quantify the concentration of the isolated DNA samples by using Spectrophotometer NanoDrop 1000 (Thermo Scientific, USA)

C. Restriction endonuclease digestion of DNA

Take out samples from the freezer

- Transfer 4.5 mg DNA samples to each new centrifuge tube and place on the ice
- Add 2.5 µl G buffer
- Add distilled water up to 25 µl
- Add 1.5 µl Pvu II
- Mix and spin down
- Incubate at 37oC in 5 hours

D. Separation of DNA fragments by electrophoresis

Make 0.8% Seakem agarose gel (Seakem GTG Agarose, Lonza, USA)

- 1.6 gram Agarose
- 200 ml TAE 1X buffer
- Add 3 µl ladder (1 kb)
- Add all 25 µl digestion of DNA
- Run program (100V in 10 minutes, 30V in 14 hours)

E. Southern Blotting

Vacuum Blotting: Vacu-Blot System (Biometra, Germany)

DNA is disengaged to simple DNA fibers and put into Hybond N+ membrane (Amersham, GE Healthcare, UK). After blotting, all fibers of DNA of gene will be transferred and fixed in membrane. UV fixed DNA to membrane

Use powder-free gloves

Cut plastic screen 19 x 19 cm (0.15 mm thick polyethylene). Cut hybond membrane 20 x 20 cm. Damp screen (a soft polyethylene) by water, put it into a square pot, smooth face on top. Put the membrane into middle of screen. Mask with 0.15 mm thick polyethylene screen to make a window appropriated with the membrane.

Take out gel from electrophoresis and put over the mask

Put square pot to the mould, close 8 clips, fit a plastic pipe in to mould, make sure it is close tight. Tight of glass bottle and start vacuum. Keep the pressure about 50 mbar

Depurination: Flush 50ml cold 0.25M HCl on the gel and make sure the depurination solution cover all gel. Keep in 7 minutes. Lean the square pot, use the glove to push out the HCL from gel. Suck out HCl. Close the pipe. Keep the pressure at 50 mbar again

Denaturalization: Flush 50ml cold 0.5M NaOH/1.5M NaCl on the gel and make sure the solution cover all gel. Check the pressure of vacuum at 50 mbar. Keep 20 minutes. Lean the square pot, push out the solution by finger. Suck out solution. Close the pipe. Keep the pressure at 50 mbar again

Neutralization: Flush 50ml cold 1M tris-HCl/1. M NaCl pH 7.5 cover all gel. Keep in 20 minutes and make sure the pressure at 50 mbar. Suck out the solution. Keep the pressure at 50 mbar again

Transfer: Fill the mould with 750ml 20X SSC until cover all the gel, avoid the gel floating. Keep 60 minutes. Make sure the vacuum pressure at 50 mbar. Suck out the solution. Discard the gel, turn off the vacuum

Place the blot on a filter paper, dry it in RT about 30 minutes. Move the blot to a new filter paper and pack it with plastic paper. UV illuminate in 5 minutes (DNA face down without plastic).

Keep in refrigerator until use

Hybridization with Probe

Pre-hybridization blocking membrane

Prepare 200 ml pre-hybridization buffer

- blocking reagent (5% w/v)
- NaCl (final concentration: 0.5M) (RT)
- Hybridization Gold buffer up to 200 ml (in refrigerator)

Mix at RT for 1 hour on a magnetic stirrer then put in the water bath at 42^0C for 0.5- 1 hour with occasional mixing

Put the blot into a clean plastic box

Cover the blot with 150ml pre-hybridization buffer and keep the rest of liquid

Place and shake the box in water bath at 42^0C for 1 hour

Hybridization (Hybridization incubators Hybrigene, Techne, UK)

Prepare hybridization solution

- 30 µl ECL labeled probe (100ng probe)
- 3 µl labeled marker (10 ng DNA ladder 1kb)
- 20 ml pre-hybridization buffer

Use a big glass tube with blue lid for hybridization

Take out the plastic box from water bath. Roll up the blot like a cigar (DNA face inside), use forceps to put the cigar blot into the tube, take out the air bubbles with a glass pipette if needed

Add hybridization solution to the tube

Place the tube to the hybridization machine

Incubate with gentle agitation overnight at 42^0C.

Wash membrane

Prepare the Primary wash buffer without urea:

- SDS 4 g (0.4% w/v)
- 20 x SSC 25 ml (final concentration 0.5 x SSC)
- Make up to 1 litre. This can be kept for up to 3 months at 2–8°C.

Prepare the Secondary wash buffer (2x SSC)

- 20 x SSC 100 ml
- Make up to 1 litre.

Preheat the primary wash buffer without urea to 55^0C

Carefully transfer the blot to a clean plastic box

Cover the blot with 200 ml primary wash buffer (without Urea) and wash twice for 10 min with mixing at 55^0C. The total wash time should not exceed 20 minutes, to protect peroxidase enzymatic activity.

Carry out a further wash in fresh primary wash buffer at 42^0C for 20 minutes.

Place the blot in a clean container

Add an excess of secondary wash buffer (about 250 ml) and wash with gentle agitation for 5 minutes at RT.

Carry out a further wash in fresh secondary wash buffer at RT for 5 minutes.

If necessary, blots maybe left in secondary wash buffer for up to 30 minutes at RT before detection.

Hybridized blots may be stored overnight wetted with secondary wash buffer in a refrigerator at 2-8^{0}C. Do not allow the blots to dry out.

Signal detection

Using Amersham ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare, UK), X-ray film cassette, Hyperfilm ECL

Mix an equal volume of detection reagent 1 (15 ml) with detection reagent 2 (15 ml) to give sufficient solution to cover the blot.

Drain the excess secondary wash buffer from the blots and place them on a sheet of cling film with DNA side uppermost.

Add the detection reagent directly to the blot on the side carrying the DNA. Do not leave the blots to dry out. Incubate for 1 minute at RT

Drain off excess detection reagent and wrap the blot in cling film. Gently smooth out air bubbles

Place the blot DNA side up in the film cassette

Switch off the lights and place on a sheet of autoradiography film (Hyperfilm ECL) on top of the blot. Close the cassette and expose for 1-5 minutes

Remove the film and develop

If required, expose a second film for an appropriate length of time (from several minutes to 2 hours)