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EPIDEMIOLOGY

Mycobacterium tuberculosis strains spreading in Hanoi, Vietnam: Beijing sublineages, genotypes, drug susceptibility patterns, and host factors

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SUMMARY

Beijing genotype strains are divided into two major sublineages, ancient (atypical) and modern (typical) types, but their phenotypic variations remain largely unknown. *Mycobacterium tuberculosis* (MTB) isolates from Hanoi, Vietnam, were analyzed by single-nucleotide polymorphisms and spoligotyping. Patient information and drug susceptibility patterns were obtained. Genetic clustering was assessed by variable number of tandem repeat (VNTR) locus sets. Multivariate analysis was also performed to investigate factors possibly associated with these sublineages. Of the 465 strains tested, 175 (37.6%) belonged to the ancient Beijing sublineage and 97 (20.9%) were of the modern Beijing sublineage. Patients with the Beijing genotype were significantly younger and more undernourished than those with non-Beijing genotype. The proportion of clustered strains calculated from 15 locus-optimized mycobacterial interspersed repetitive units [optimized-(MIRU)15]-, optimized-MIRU24-, optimized-MIRU28-, Japan Anti-Tuberculosis Association (JATA)15-, and JATA18-VNTRs were 55.7%, 49.2%, 33.8%, 44.5%, and 32.0%, respectively. Ancient and modern Beijing genotype strains were more frequently clustered than non-Beijing genotype strains, even when using VNTR sets with high discriminatory power. Isoniazid and streptomycin resistance tended to be more frequently observed in ancient Beijing strains than in modern Beijing strains and others. Our findings may provide insight into area-dependent differences in Beijing family strain characteristics.

1. Introduction

Tuberculosis remains a major public health problem, with an estimated 8.8 million cases and almost 1.4 million deaths occurring annually worldwide [1]. The global population structure of the pathogen *Mycobacterium tuberculosis* (MTB) is currently defined by seven major lineages, of which the Beijing genotype family belongs to the East-Asian lineage [2]. This genotype represents more than 50% of strains in East Asian areas [3].

Studies report that Beijing genotype strains are becoming widespread, even outside Asia. It is possible that this occurs through the exploitation of an imperfect host immune system or it

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may be associated with antibiotic resistance, including multidrug-resistant TB [4,5]. However, the results of these studies are not always consistent, and phenotypic variations in major subtypes within the Beijing genotype remain largely unknown [5]. *In vitro* studies [6] and evidence from studies using animal models [7] have shown that the hypervirulence displayed by Beijing genotype strains is not common to all members of the Beijing family but is restricted to some subsets of the strains.

Beijing genotype strains are divided into two major sublineages, ancient (atypical) and modern (typical), according to the absence or presence of an IS*6110* insertion, respectively, in a particular chromosomal position designated as the NTF region [8]. Modern Beijing genotype strains are prevalent around the northern area of mainland China and extend to the former Soviet Union and South Africa [9], while ancient Beijing genotype strains are predominant in Japan and Korea [10–12].

Vietnam is a Southeast Asian country stretching over 1800 km from north to south. To the south of Vietnam, the Beijing genotype strains seem to be predominant in hospital settings, ranging from 53% [13] to 82% [14], but have been shown by population-based studies to be less predominant in rural areas (35.6% [15]). So far, except for one study conducted on the proportion of sublineages among the Beijing family in the south of Vietnam [16] from 1998 to 1999, there have been no comprehensive studies focused on the phenotypic variations of the ancient and modern Beijing genotypes in this country. Thus, we investigated the prevalence and characteristics of MTB lineages and sublineages in the north of Vietnam (Hanoi, the capital city) circulating among patients newly diagnosed with pulmonary TB and identified factors possibly associated with the Beijing genotypes. To confirm which sublineage of the Beijing genotype strains is predominant, we also tested another set of MTB samples isolated in Hanoi.

It is also important to know the clustering information of the MTB isolates in this area as it may indicate recent transmission events [17,18]. To investigate the genetic clustering of the MTB lineages and sublineages, we tested different locus sets of the variable number of tandem repeat (VNTR) genotyping system: These included two international standard typing systems; the 15 and 24 locus sets (optimized-mycobacterial interspersed repetitive units [MIRU] 15- and -MIRU24-VNTR) and three others recommended for the Beijing genotype strains; a new set (optimized-MIRU28-VNTR) consisting of 24 loci of the optimized-MIRU24 plus four additional loci [VNTR-1982 and three hypervariable (HV) loci (VNTR-3232, -3820, and -4120)], which was recently recommended by the Pasteur Institute in France [19], the Japan Anti-Tuberculosis Association (JATA)15-VNTR set consisting of JATA12-VNTR [20] plus three loci (ETR-A, VNTR-1982, and -2163a), and the JATA18-VNTR set consisting of JATA15 plus the three aforementioned HV loci, of which the JATA12 or 15 system has been integrated into the TB control system nationwide and is currently used in Japan, where Beijing strains are predominant [21]. We also assessed the performance of these systems.

2. Materials and methods

2.1. Study sites, recruitment of patients, and ethics statement

MTB isolates were collected as a part of a cohort study [22]. Written informed consent was obtained from each participant. In the case of minors, their parents provided written informed consent. This study was approved by the Ethical Committees of the Ministry of Health, Vietnam, National Center for Global Health and Medicine, and the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan.

From July 2007 to March 2009, seven districts of Hanoi city in Vietnam were enrolled as a study area, and approximately 70% of patients diagnosed with new smear-positive pulmonary TB in the districts agreed to participate in this study. All the participants were Vietnamese. They received the standard 8-month regimen of 2S(E)HRZ/6HE, which was commonly administered during the study period in Vietnam. In the current study, only one isolate per patient at the time of diagnosis was used for analysis. We also tested another set of DNA samples that were consecutively collected using mycobacteria growth indicator tubes (MGIT) in the Hanoi Lung Hospital in 2011.

2.2. Identification of MTB, drug susceptibility testing, and molecular genotyping

Identification of MTB, and drug susceptibility testing to isoniazid (INH), streptomycin (SM), rifampicin (RMP), and ethambutol (EMB) were performed as reported before [22]. Beijing and non-Beijing strains were analyzed by single-nucleotide polymorphism (SNP) at position 779,615 [23] using real-time PCR with a TaqMan MGB probe [Primers, mtb779615-F: CGATTGGCCTGTGGTCACT; mtb779615-R: GAACAACAAGATCGCCTTCGA; Probe, wild-type (FAM): TAGGTGACCGTGTTGTC; mutant (VIC): TAGGTGACCGTCTTGTC]. Ancient and modern Beijing genotypes were identified by PCR, the conditions and analysis of which were described previously [24]. Spoligotyping was performed [25] in parallel and their genotypes were identified using the international MTB database (SpolDB4) [26].

Five different VNTR locus sets were tested for the identification of genotypic clusters: optimized-MIRU15-, optimized-MIRU24-, optimized-MIRU28-, JATA15-, and JATA18-VNTR sets (Supplementary Table S1). Amplified products were analyzed using a 3130 Genetic Analyzer (ABI) with the GeneMapper program, SV1210 microchip electrophoresis (Hitachi) or agarose gel electrophoresis. The copy number in each locus was calculated based on the molecular size of the PCR products and the number of tandem repeats in the genome of the H37Rv strain was used as the standard.

Genetically clustered strains were defined by the complete match of the VNTR profile. To confirm the appropriateness of each cluster, spoligotyping patterns were also considered. The proportion of clustered strains (the clustered proportion) was calculated using the "*n*" method, which is given by the number of isolates in clusters divided by the total number of isolates [18]. Polymorphic information content (PIC) was used as one of the estimators for the discriminatory power of typing loci [27]. Also, genetic diversity and discriminatory power were assessed by calculating the number of different VNTR patterns and the Hunter–Gaston discriminatory index (HGI) for each condition [28]. Using VNTR profiles of MTB isolates by optimized-MIRU15-, optimized-MIRU28-, JATA15-, and JATA18-VNTR systems, the minimum spanning trees were depicted using BioNumerics software version 4.61 (Applied Maths).

2.3. Statistical analysis

The chi-square test was used to compare the proportions between groups. Bonferroni's correction was used for multiple comparisons. Median and interquartile range (IQR) were presented for age distributions and the Kruskal–Wallis test was used to assess their possible differences among MTB subtypes. Polytomous logistic regression models for MTB lineages or sublineages as outcome variables were also used to investigate factors showing associations, after which adjusted odds ratios (aORs) and 95% confidence intervals (CIs) were calculated. Interaction terms were also considered when appropriate. Factors with biological meaning or showing $P < 0.2$ in univariate analysis were included in multivariate models. The McNemar's test was used to investigate a

possible inconsistency of the power to detect unique (non-clustered) strains between two VNTR sets, which tests whether the frequency of unique strains detected by one VNTR set is significantly different from that of another set. Statistical analysis was performed using Stata version 12 (Stata Corp, College Station, TX, USA), and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of the study population

For 465 MTB strains, the characteristics of the study population by MTB lineages, including Beijing sublineages, are provided in Supplementary Table S2. In summary, the median patient age was 39.0 years (IQR 29.2–50.6); 365 (78.5%) patients were male and 38 (8.2%) were HIV-positive.

3.2. Genotypes of MTB strains defined by SNPs and spoligotyping in Hanoi

Table 1 shows the proportions of ancient/modern Beijing and non-Beijing genotypes identified by the methods of SNPs and spoligotyping. Of the 465 strains tested, 175 (37.6%) belonged to the ancient Beijing sublineage and 97 (20.9%) were of the modern Beijing sublineage. Thus, the total proportion of the Beijing lineage strains was 58.5%. The third most prevalent type was East African Indian (EAI), which usually belongs to the Indo-oceanic lineage. Other spoligotypes, including H, LAM, T, U, and X, were seen in 65 cases (14.0%). In 37 cases (8.0%), spoligotypes were not registered in the SpolDB4 database. Thus, the spoligotyping method was limited in its ability to identify and classify genotypes.

When we tested another set of 223 DNA samples in Hanoi, the ancient Beijing sublineage was reproducibly predominant. Of these samples, 88 (39.4%) belonged to the ancient Beijing sublineage, 61 (27.4%) were of modern Beijing sublineage, and 74 (33.2%) were non-Beijing strains.

3.3. Patient characteristics stratified by ancient and modern Beijing sublineages, EAI genotype, and other strains

Ancient and modern Beijing genotype strains were both widespread among young patients [median age was 37.9 years (IQR, 29.2–49.1) and 34.8 years (IQR, 28.5–48.8), respectively], while EAI strains were frequently seen in a relatively older group [46.8 years (IQR, 33.1–56.3)] (Supplementary Table S2). The age distribution was significantly different among the four groups even after Bonferroni's correction (uncorrected $P = 0.0036$, Kruskal–Wallis test). When we investigated additional demographic parameters or clinical features such as lesions on chest radiography or HIV status by the chi-square test, the proportions of subcategories in each parameter or feature were not significantly different among the four groups (Supplementary Table S2).

Table 1
Frequency of the MTB genotype defined by SNPs and spoligotyping ($n = 465$).

Spoligotypes*	Frequency	Percentage
Beijing (ancient type)	175	37.6
Beijing (modern type)	97	20.9
EAI5	82	17.6
H1	3	0.7
H3	9	1.9
H4	1	0.2
LAM9	2	0.4
MANU2	1	0.2
S	2	0.4
T1	31	6.7
T2	4	0.9
T2-T3	4	0.9
T3	1	0.2
U	5	1.1
EAI4_VNM	9	1.9
X1	1	0.2
X2	1	0.2
Unknown	37	8.0

MTB: *Mycobacterium tuberculosis*.
* Beijing/non-Beijing and ancient/modern Beijing genotypes were classified by SNPs.

3.4. Genetic clustering based on VNTR typing methods using different locus sets

Because it is well known that the genetic clusters of Beijing genotypes are not clearly identified by the optimized-MIRU15- or MIRU24-VNTR typing systems [4], we added three different locus sets for the calculation of the clustered proportions: JATA15-and JATA18-VNTR locus sets and the recently proposed 24 plus 4 locus sets, optimized-MIRU28 system, considering clonal spread of Beijing genotypes.

Of the 465 strains, the proportions of clustered strains calculated from the optimized-MIRU15-, optimized-MIRU24-, and JATA15-VNTR sets were 259 (55.7%), 229 (49.2%), and 207 (44.5%), respectively. Because differences in these percentages are mainly attributed to the differences in discriminatory power to identify unique VNTR patterns, we compared the proportion of unique (nonclustered) strains detected by the JATA15 set and demonstrated that it was significantly higher than that of the MIRU15 or MIRU24 sets (data not shown; $P < 0.0001$ and $P = 0.0068$, respectively, by the McNemar's test). Based on the optimized-MIRU28 locus set, including three HV loci (VNTRs-3232, -3820, and -4120) [19], the proportion of clustered strains was 157 (33.8%), which was relatively lower than those of the aforementioned sets. As expected, the proportion calculated from the JATA18 set (JATA15 plus the same HV loci) was similarly low at 149 (32.0%) and not inferior to that of the MIRU28 system ($P = 0.1573$).

Ancient and modern Beijing genotype strains were more frequently clustered than the EAI genotype, even when considering multiple comparisons (uncorrected $P < 0.0001$, Supplementary Table S2). We also calculated the proportion clustered in each (sub)lineage using five different VNTR locus sets (Figure 1). In both the ancient and modern Beijing MTBs, the JATA sets tended to show a reduced proportion of clustered strains, presumably indicating high discriminatory power as compared with the MIRU systems using the equivalent number of VNTR loci. In contrast, this advantage of JATA sets was not observed for non-Beijing sublineages such as the EAI spoligotype (Figure 1). A similar tendency was obtained when the genetic diversity of MTB (sub)lineages and the discriminatory power of each VNTR set were assessed by the number of different VNTR patterns (=the number of unique strains plus one from each cluster), the proportion of unique strains, and the Hunter–Gaston Index (HGI) (Supplementary Table S3).

Because the discriminatory power of VNTR methods to assess MTB's clonality and transmission is largely affected by the MTB (sub)lineages analyzed, we used the minimum spanning tree program and further analyzed the relatedness of the Beijing and non-Beijing strains genotyped by the different locus sets (Supplementary Figure S1). Non-Beijing MTBs were subdivided into two large groups in both optimized-MIRU15- and optimized-MIRU28-VNTR systems. As compared with these MIRU systems, JATA15-and 18-VNTRs clearly separated the modern and ancient Beijing sublineages into two different groups, although these locus sets did not show a sufficient power to further divide non-Beijing MTBs.

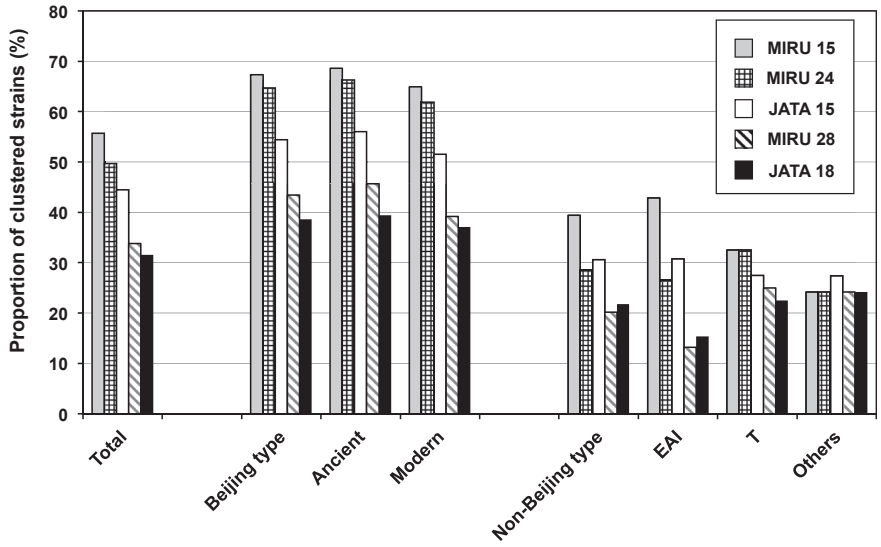

Figure 1. The proportion of clustered strains determined in each (sub)lineage using different VNTR locus sets. Using optimized-MIRU15, 24, 28, JATA15, and 18 locus sets, 465 MTB isolates were analyzed. The proportions of clustered strains were calculated.

3.5. Profiles of drug resistance harbored by ancient and modern Beijing sublineages, EAI genotype, and other strains

Next, we examined the relationship between drug resistance and the MTB genotypes. The proportions of strains carrying "any INH resistance" and "any SM resistance" tended to be high in the ancient Beijing genotype group, lower in the modern Beijing genotype group, and the lowest in the EAI genotype group. The differences in the proportion of INH- and SM-resistant strains among the four MTB genotype groups, including others, were statistically significant, even after considering multiple comparisons (uncorrected $P = 0.0001$ or <0.0001 by the chi-square test, respectively, Table 2). No significant difference was observed in the proportions of RMP, EMB, or multidrug resistance among these groups (Table 2).

Table 2
MTB sublineages and patterns of drug resistance ($n = 465$).

Drug resistance	Number (%) of isolates with drug resistance in the different groups of MTB genotype				
	Ancient Beijing $N = 175$	Modern Beijing $N = 97$	EAI $N = 91$	Others $N = 102$	P value*
Sensitive to all drugs	83 (47.4)	66 (68.0)	78 (85.7)	60 (58.8)	<0.0001
Any INH resistance	69 (39.4)	23 (23.7)	13 (14.3)	23 (22.6)	0.0001
Any RMP resistance	11 (6.3)	5 (5.2)	1 (1.1)	4 (3.9)	0.26
Any SM resistance	70 (40.0)	19 (20.0)	5 (5.5)	32 (31.4)	<0.0001
Any EMB resistance	5 (2.9)	2 (2.1)	0 (0.0)	4 (3.9)	0.307
INH monoresistance	21 (12.0)	11 (11.3)	8 (8.8)	8 (7.8)	0.671
RMP monoresistance	1 (0.6)	0 (0.0)	0 (0.0)	1 (1.0)	>0.999
SM monoresistance	21 (12.0)	8 (8.3)	0 (0.0)	18 (17.7)	0.0005
INH + SM	38 (21.7)	7 (7.2)	4 (4.4)	11 (10.8)	<0.0001
INH + EMB	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	0.624
SM + EMB	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
INH + RMP	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)	0.404
INH + RMP + SM	6 (3.4)	2 (2.1)	1 (1.1)	0 (0.0)	0.223
INH + RMP + EMB + SM	4 (2.3)	2 (2.1)	0 (0.0)	3 (2.9)	0.503

MTB: *Mycobacterium tuberculosis*; EAI: East African Indian; INH: isoniazid, RMP: rifampicin, SM: streptomycin, EMB: ethambutol.

* P value for an overall difference in the proportions of drug-resistant strains stratified by the four MTB subgroups was calculated using the chi-square test. Underlined values indicate that the statistical significance remained after Bonferroni's correction.

3.6. Characteristics associated with ancient and modern Beijing sublineages

To further clarify the phenotypic characteristics of ancient and modern Beijing sublineages, we compared them with non-Beijing strains, including EAI strains and others. By univariate analysis (Table 3), patients younger than 55 years old were associated with both ancient and modern Beijing strains. Relatively low BMI levels (16.0–18.4) also showed an association with ancient and modern Beijing genotypes. Herein, we used the optimized-MIRU28-VNTR system and defined the genetic clusters, which were strongly associated with ancient and modern Beijing strains. All the above

Table 3
Univariate analysis using polytomous logistic regression models to investigate factors possibly associated with MTB sublineages ($n = 465$).

	Odds ratio* (95% CI)	
	Ancient Beijing	Modern Beijing
Age: <55.0 years vs. ≥55.0 years	**3.38 (1.84-6.21)**	**2.66 (1.31-5.39)**
Female vs. male	1.17 (0.70–1.94)	1.46 (0.82–2.61)
Body mass index		
<16.0 vs. no undernutrition†	1.12 (0.60–2.10)	1.01 (0.46–2.24)
16.0–18.4 vs. no undernutrition	**1.60 (1.03-2.51)**	**1.85 (1.09-3.13)**
Living area		
New urban vs. suburban	0.99 (0.56–1.73)	0.61 (0.33–1.14)
Old urban vs. suburban	0.91 (0.50–1.63)	0.53 (0.27–1.02)
Smoking vs. nonsmoking	0.83 (0.54–1.29)	0.78 (0.47–1.31)
HIV (+) vs. HIV (−)	1.20 (0.56–2.56)	1.30 (0.54–3.12)
Cavity vs. no cavity on CXR	0.91 (0.58–1.43)	1.52 (0.85–2.71)
Infiltrates in ≥3 vs. <3 lung zones on CXR	1.29 (0.74–2.26)	1.31 (0.68–2.51)
Clustered vs. nonclustered§	**3.33 (2.10–5.27)**	**2.54 (1.48–4.36)**
INHr vs. INHs	**2.84 (1.77–4.55)**	1.36 (0.75–2.45)
SMr vs. SMs	**2.81 (1.76–4.49)**	1.03 (0.55–1.90)
RMPr vs. RMPs	2.52 (0.86–7.41)	2.04 (0.58–7.24)

MTB: *Mycobacterium tuberculosis*, HIV: Human immunodeficiency virus, CXR: chest radiography, INHr: resistant to isoniazid, INHs: sensitive to isoniazid, SMr: resistant to streptomycin, SMs: sensitive to streptomycin, RMPr: resistant to rifampicin, RMPs: sensitive to rifampicin, 95% CI: 95% confidence interval.
Boldfaced values indicate odds ratios and 95% CI with statistical significance ($P < 0.05$).

* The non-Beijing group including EAI strains was set as a reference.

† No undernutrition indicates a body mass index over 18.5.

§ Genetic clustering was defined on the basis of the optimized-MIRU28-VNTR system.

associations remained significant in multivariate analysis using the model including age, gender, BMI, presence of cavity on chest radiography, clustering status, and resistance to INH, SM, and RMP (Table 4).

We also analyzed the interaction term between genetic clustering and drug resistance and the independent effects of each category. By univariate analysis, INH resistance appeared to be associated with ancient Beijing strains (Table 3), but this association was lost after adjustment for age and other possible confounders (Table 4). The interaction term between INH resistance and genetic clustering did not show particular effects on ancient Beijing strains, whereas it showed significantly negative effects on modern Beijing strains, even after adjustment for possible confounders (Table 4).

SM resistance was also associated with the ancient Beijing strains in a univariate analysis (Table 3). This association was lost (aOR = 1.95, 95% CI 0.97–3.91) after adjustment for age and other possible confounders when genetic clustering was defined by optimized-MIRU28-VNTR (Table 4). When the clusters were defined by the JATA18-VNTR set, both INH and SM resistance showed weak but significant associations with the ancient Beijing strains after adjustment (aOR = 2.15, 95% CI 1.00–4.61 and aOR = 1.98, 95% CI 1.00–3.92, respectively) (data not shown). The interaction term between SM resistance and genetic clusters also tended to show slightly negative effects on modern Beijing strains, but it did not reach significant levels (Table 4). RMP resistance was not associated with either ancient or modern Beijing strains, and we could not attempt further analysis due to insufficient statistical power.

The relationship between BCG vaccination and the MTB subtype was not included in logistic regression analysis and was analyzed separately because of the large proportion of missing values. As a result, no significant associations were observed between these two factors (data not shown).

Table 4
Multivariate analysis using polytomous logistic regression models to investigate factors possibly associated with MTB sublineages ($n = 465$).

	Odds ratio* (95% CI)	
	Ancient Beijing	Modern Beijing
Age: <55.0 years vs. ≥55.0 years	**3.44 (1.70–6.98)**	**2.19 (1.03–4.66)**
Female vs. male	1.42 (0.79–2.55)	1.62 (084–3.10)
Body mass index:		
<16.0 vs. no undernutrition†	1.32 (0.64–2.73)	0.91 (0.38–2.19)
16.0–18.4 vs. no undernutrition	**1.86 (1.13–3.07)**	**2.00 (1.13–3.54)**
Cavity vs. no cavity on CXR	0.87 (0.53–1.42)	1.44 (0.78–2.64)
Clustered vs. nonclustered§	**3.28 (1.70–6.33)**	**4.32 (2.17–8.62)**
INHr vs. INHs	1.83 (0.85–3.92)	2.03 (0.84–4.89)
SMr vs. SMs	1.95 (0.97–3.91)	0.93 (0.39–2.24)
RMPr vs. RMPs	1.11 (0.32–3.85)	2.11 (0.49–9.09)
Interaction between clustering and INHr**	0.44 (0.13–1.46)	**0.15 (0.03–0.66)**
Interaction between clustering and SMr**	1.23 (0.37–4.13)	0.56 (0.11–2.91)

MTB: *Mycobacterium tuberculosis*, CXR: chest radiography, INHr: resistant to isoniazid, INHs: sensitive to isoniazid, SMr: resistant to streptomycin, SMs: sensitive to streptomycin, RMPr: resistant to rifampicin, RMPs: sensitive to rifampicin, 95% CI: 95% confidence interval. For assessment of the interaction between clustering and drug resistance, the main effects with an interaction term were included in a polytomous logistic regression model.

Boldfaced values indicate odds ratios and 95% CI with statistical significance ($P < 0.05$).

* The non-Beijing group including EAI strains was set as the reference.

† No undernutrition indicates a body mass index over 18.5.

§ Genetic clustering was defined on the basis of the optimized-MIRU28-VNTR system.

** A full factorial model was developed; both interaction terms and independent effects are shown.

4. Discussion

Our study showed that MTB strains of ancient and modern Beijing genotypes consisted of the largest and the second largest groups circulating among patients newly diagnosed with smear-positive, culture-positive pulmonary TB in Hanoi, Vietnam. Age distribution, genetic clustering, and the patterns of primary drug resistance were differently dependent on MTB genotypes, including Beijing sublineages. This was the first study in the northern part of Vietnam that investigated the phenotypic characteristics of Beijing sublineages.

In our study population, Beijing genotype strains accounted for 58.5% of MTB strains, comparable with that of East Asian areas [26]. This prevalence is higher than that reported from rural Vietnam, where EAI strains are more predominant [29]. EAI belongs to the Indo-oceanic lineage, one of the most ancestral of the seven MTB lineages [2]. EAI strains may have originated from Africa [30] and spread to the Southeast Asian area accompanied by the population movement through the southern regions of Eurasia. These strains may have gradually been replaced by the recent expansion of the Beijing genotype strains [29]. This hypothesis is worth considering and should be tested by monitoring MTB (sub)lineage distribution in Hanoi for an extended timeframe. A long-term study is required because the Beijing genotype is more commonly seen in younger populations and is clustered compared with those infected with the EAI genotype and others in this study area; this suggests the possibility of recent spread of the Beijing genotype.

Of the Beijing genotype strains in Hanoi, located in the northern part of Vietnam, the ancient sublineage accounted for two-thirds and the modern sublineage one-third. Interestingly, this distribution pattern is similar to that from Japan [10,11] and Korea [12], but this pattern is different from the patterns reported from most other parts of the world, such as China [31], Russia [32], South Africa [9], and Europe [16], where the modern Beijing genotype represents 65%–95% of Beijing strains. Another study from Ho Chi Minh city in southern Vietnam also has showed that the modern Beijing genotype is observed three times more frequently than the ancient Beijing subtype [16]. This difference in distribution between these two major cities at the far ends of Vietnam may be due to the northern part of Vietnam bordering on southern China, where ancient Beijing is also more frequently found as compared with the northern areas of China [31]. In addition, in our study population, the proportions of both the ancient and modern Beijing sublineages were higher in younger patients, suggesting their recent dissemination. This finding is in contrast to the ancient type spreading among older patients in the southern part of Vietnam [16]. Further information regarding sublineage distribution throughout many Asian countries is necessary to approach the evolutionary history, including a potential branching point between the ancient and modern Beijing genotypes. Thus far, little information regarding these sublineages is available in Southeast Asia, including Vietnam [5].

Because Beijing genotype isolates are closely genetically related to each other, many genotyping methods exhibit low discriminatory power and a limited potential to assess their genetic clonality that reflects epidemiological transmission [19,33]. In our tested population, the discriminatory power of JATA15 (a local Japanese system used in a Beijing genotype-predominant area) was higher than that of optimized-MIRU15 or 24, in which all worldwide lineages are the targets. When appropriate HV loci were added to either the optimized-MIRU24 or JATA15 set, the genotyping systems were more suitable for the Beijing family. Considering the resource-poor settings in many Asian countries, however, it is difficult to analyze more than 20 genetic loci for domestic public health problems with the exception of international research

activities. For instance, in Japan, 70%–80% of MTB isolates are of the Beijing genotype [11], and 12 or 15 VNTR loci have been preferred on site [20]. A similar cost-effective VNTR locus-set has also been recommended in China [34]. Despite the relatively small number of tested loci, in our study, the Japanese system had high discriminatory power for MTBs in the northern part of Vietnam. PIC for one of the HV loci, VNTR-4120, in Japan and Thailand was reportedly 0.90 and 0.58, respectively [27,35]. In our study, PIC for the VNTR-4120 locus in Hanoi was 0.83 (data not shown). This finding may indicate that the distribution patterns of the Beijing MTB genotypes in Hanoi resemble those in Tokyo, including both the ancient and modern sublineages. Considering the proportion of unique (non-clustered) strains and other indexes, including HGI, it appears that MIRU28 and JATA18 have a higher discriminatory power than the others. However, a drawback of adding the HV loci is that a large number of nucleotide repeats in the loci should be distinguished using a high-resolution genetic analyzer. Although it is conceivable that TB transmission was ongoing during the study period in Hanoi districts in which patients were recruited, direct information regarding transmission chains between clustered cases or the possible involvement of outbreak strains was not available, which is a limitation of our study. Minimum essential VNTR loci optimal to TB transmission should be further examined in a prospective population-based study and discussed with information about the epidemiological link.

Even when we used VNTR locus sets with a high discriminatory power, Beijing genotype strains were frequently clustered, whereas the majority of the EAI genotype and other strains were observed as nonclustered strains. The associations between the Beijing sublineages and clustering remained significant, even after adjustment for other factors in a polytomous regression model. In our study area, the modern Beijing genotype strains were less prevalent than the ancient Beijing genotype strains, while the proportion of clustered strains belonging to the modern Beijing genotype was comparable to that of the ancient Beijing genotype, irrespective of the different VNTR loci sets. Although we have no direct evidence, the modern Beijing strains may have the potential to spread further in this area. Indeed, previous reports have often shown that these strains have a high transmissibility [36,37].

Associations between the antibiotic resistance and Beijing genotype strains have also been investigated in many studies in various geographical settings [3–5], although the results are controversial. Interestingly, the interaction between INH resistance and genetic clustering was significantly less likely to occur in the modern Beijing strains, irrespective of possible confounders in our study. Although Beijing genotype strains are often spreading as MDR-TB (INH and RMP resistance) in many areas worldwide, the clustered modern Beijing genotype strains identified in the Hanoi area may belong to some different subgroup(s) with a tendency to spread without harboring INH-resistance. One possibility is that these strains may be disadvantageous to propagation once they acquire drug resistance, bearing a higher “fitness cost” than those widely spreading in other areas. A detailed comparative analysis is necessary to better understand this issue, possibly analyzing genome-wide variations among several subgroups of the modern Beijing sublineage. Another possibility is that a majority of modern Beijing sublineage strains in Hanoi may have recently entered across neighboring countries as drug-susceptible strains and may currently be spreading in Hanoi.

Also, in our study, the ancient Beijing strains in Hanoi tended to carry INH and SM resistance more frequently than the modern Beijing strains and others. However, this association was not always significant, but it was affected by other factors such as the age of the patients in the multivariate analysis. The tendency of the ancient Beijing strains to carry drug resistance has also been demonstrated in a few reports from East Asian areas where the Beijing strains are predominant [21,38]. However, the drug resistance patterns of the ancient Beijing genotype strains were different: INH and RMP in one report [21] and pyrazinamide and RMP in another [38]. These differences may be relevant to the history of when the antibiotics were introduced or because of other confounders. In Vietnam, SM was initially used for the treatment of wound infections during the war in the early 1950s, after which INH was widely implemented for tuberculosis treatment, which may partly explain the current spread of SM- and INH-resistant strains. Depending on drug resistance, the fitness of the ancient Beijing genotype strains may be retained or may even be stronger than the modern Beijing strains. We revealed that 116 (84.1%) of 138 INH-resistant strains identified in this study harbored a single *katG* S315T mutation (unpublished data), which seems to have a negligible fitness cost, thus indicating no reduction in transmissibility [39,40]. Further study is necessary to elucidate whether bacterial genetics have an epistatic impact on propagation of drug resistance through the genotype to which they belong [39].

Both ancient and modern Beijing strains were more likely to be detected from relatively undernourished patients (~60%), whereas more than half of EAI strains were observed in patients with normal BMI. This association remained significant even after adjustment for possible confounders. Severe undernutrition with a BMI less than 16.0 did not show significant association, probably due to the small number of cases or different reasons. Malnutrition itself may be a condition that makes patients vulnerable to infections by the Beijing genotype strains, or it may be brought on by infection with the MTB strains [41]. The relationship between host nutritional state and activation of modern/ancestral MTB lineages would be one of the important topics to consider in the host–pathogen interaction and future therapeutic modalities.

Although it is difficult to adjust for the historical flow of MTB strains introduced from outside areas, potential confounders to the interpretation of the genotype–phenotype relationship of the MTB strains were minimized in our study. Both the ancient and modern Beijing genotype strains were commonly observed with non-Beijing strains among the Vietnamese population with relatively homogeneous ethnicity [42]. This indicates that the northern part of Vietnam may be one of the suitable geographic areas to characterize these Beijing sublineages as compared with the non-Beijing strains.

In conclusion, our study showed that among patients newly diagnosed with smear-positive, culture-positive pulmonary TB in Hanoi, Vietnam, ancient Beijing genotype strains are predominant, followed by the modern Beijing sublineage. Both appear to be currently spreading; however, their phenotypes are different, even though they both belong to the same Beijing family. Our findings may provide an insight into the reason(s) for inconsistencies among previous results regarding the overall phenotypic characteristics of the Beijing family.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2014.09.005.

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