NK cells, CD8+ T cells, granulysin, perforin and IFN-γ in patients with HIV/TB coinfection and TB

The number of NK, *i*NKT, Vγ9+Vδ2+ T, CD4+ T and CD8+ T cells were correlated with granulysin, perforin, granzyme-B and IFN-γ levels at the time of enrollment. In HIV/TB patients, NK cells and CD8+ T cells were not significantly correlated with granulysin, but both cell types positively correlated with perforin ($p = 0.045$, $r = 0.714$ and $p = 0.036$, $r = 0.771$, respectively). In TB patients, NK cells showed negative correlation, whereas CD8+ T cells was positively correlated with granulysin ($p = 0.011$, $r = -0.499$), $p = 0.049$, $r = 0.398$, respectively). For IFN-γ, a trend to increase in relation to the numbers of NK cells in patients with both HIV/TB coinfection and TB were seen. For the rest, no significant correlations were found among effector molecules and cell populations.

Increased circulating granulysin and decreased IFN-γ levels in HIV/TB coinfection and TB after completion of anti-TB therapy

After 6-9 months of anti-TB therapy, only 3 HIV/TB patients and 13 TB patients could be followed-up when they were considered as treatment success (Figure 4). In patients with HIV/TB coinfection, the granulysin levels after completion of anti-TB therapy (median = 8.535 ng/ml) showed a trend to increase compared to those before therapy (median = 7.365 ng/ml) ($p = 0.208$), although significant difference was not found (Figure 4A). Whereas in TB patients, the granulysin levels after completion of anti-TB therapy (median = 1.861 ng/ml) were significantly higher than those before therapy (median = 1.048 ng/ml) ($p = 0.002$) (Figure 4B).

In contrast, the IFN-γ levels after completion of anti-TB therapy were significantly lower in HIV/TB (median <4.7 pg/ml) (Figure 4C) and TB patients (median <4.7 pg/ml) (Figure 4D) than those before therapy (median = 53.04 pg/ml for HIV/TB, $p = 0.037$ and 10.04 pg/ml for TB, $p = 0.012$).

Clinical profiles in relation to effector molecules and cells in patients with HIV/TB coinfection

Among 6 HIV/TB patients, 4 were considered as cured, whereas 2 could not be followed-up (Table 1). Among these, 3 patients with cured after 6-8 months of TB treatment had high granulysin and perforin levels and high number of NK cells (Table 2). Obviously, they had very high CD8+ T cells. While one patient with cured after 18 months of TB treatment had quite low CD8+ T cells, but high NK cells and even very high IFN-γ levels, but the granulysin and perforin levels were lower than other 3 patients.

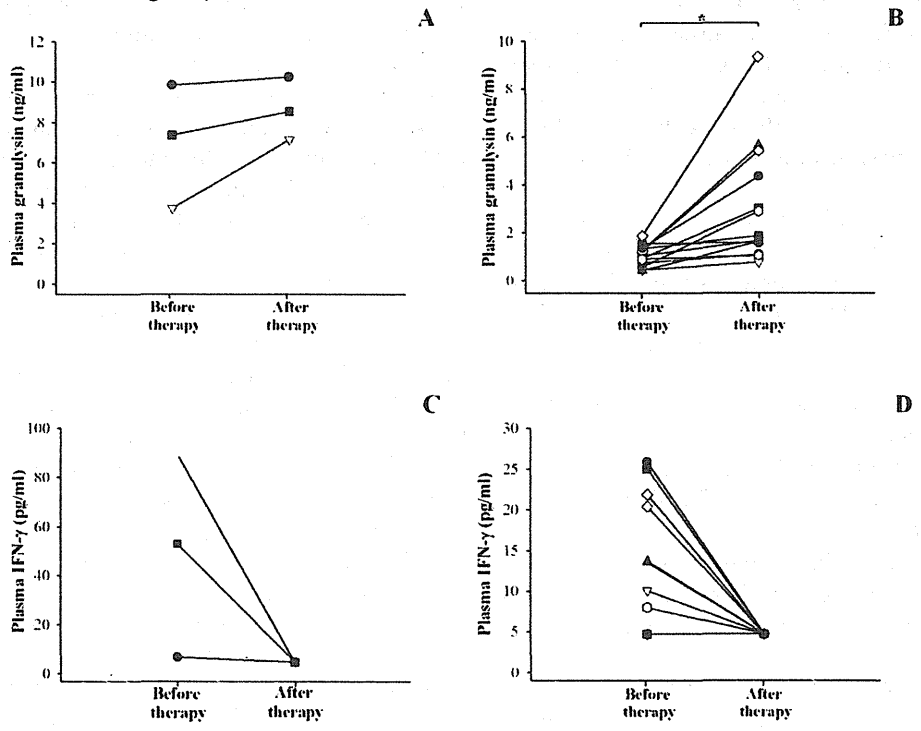

Fig 4. Circulating granulysin (ng/ml) and IFN-γ (pg/ml) in Thai patients with HIV/TB coinfection (A and C) and patients with TB (B and D) before and after completion of anti-TB therapy. Each dot represented one individual. *, $p < 0.05$.

Discussion

In this study, circulating granulysin and other immune molecules, perforin, granzyme-B and IFN-γ in relation to the numbers of NK cells, *i*NKT cells, Vγ9$^+$Vδ2$^+$ T cells, CD4$^+$ T and CD8$^+$ T cells in HIV/TB coinfection, TB and other control groups before and after completion of anti-TB therapy were investigated. Before anti-TB therapy, the extremely higher granulysin in HIV/TB coinfection and slightly lower granulysin in active pulmonary TB than HC were noted, and both increased after completion of anti-TB therapy, presumably indicating its protective role of host defense against *Mtb* infection. Low granulysin levels in active TB, may be explained by rapid consumption due to ongoing effector immune response, or reduced during active disease due to the reduction of T cell subsets dedicated to its production [12, 25]. Interestingly, the results of higher granulysin and perforin levels, higher number of NK cells and obviously higher number of CD8$^+$ T cells in HIV/TB coinfection with cured after 6-8 months of TB treatment than the one with cured after 18 months upon therapy, indicated the effective role of NK cells in innate and CD8$^+$ T cells in adaptive immunity. However, the patient with 18 months cured had high NK cells and obviously high IFN-γ levels suggesting the effective role in innate immunity. These results in Thai patients with HIV/TB coinfection were in accordance with the findings in Japanese patients with HIV/TB coinfection which also showed the higher plasma granulysin levels (median = 15.222 ng/ml, ranged 11.372-19.946, n = 19) than healthy individuals (median = 4.869 ng/ml, ranged 2.262-9.983, n = 19) (Figure 5) (Data provided by Dr. Shinichi Oka and his colleagues, AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan on November 1st, 2012). The explanation of the high granulysin in HIV/TB coinfection might be possibly due to (i) exposure to many antigens or with complication occurrences once those patients were concurrently infected with both pathogens as chronic infection which may influence quite high granulysin levels or (ii) the results of immune activation in HIV/TB coinfection or (iii) high number of CD8$^+$ T cells may play a major cell for granulysin production.

In this study, three bands with ~17 kDa, 15 kDa and 9 kDa were identified in plasma from HIV/TB coinfected patients. Though 9 kDa form of granulysin is cleaved from 15 kDa precursor by protease, it is known that 9 kDa form cannot be detected in normal plasma [10]. It is assumed that ~17 kDa band may correspond to full length granulysin with signal sequence. Release of granulysin with signal peptide is questionable, except that from disrupted cells due to necrosis. So far, this is the first demonstration of granulysin in patients with HIV/TB coinfection using DS method to concentrate peptides and low molecular weight proteins in plasma.

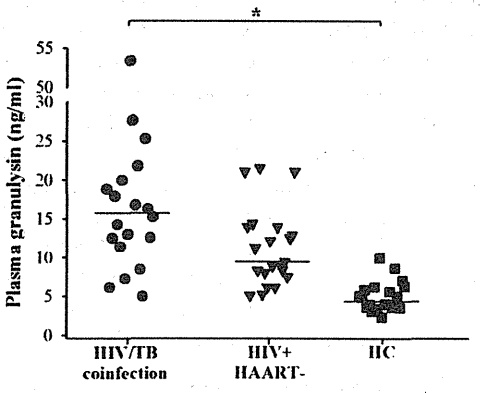

Fig 5. Circulating granulysin (ng/ml) in Japanese patients with HIV/TB coinfection before anti-TB therapy, HIV+HAART- and healthy controls (HC). Each dot represented one individual. A horizontal bar indicated the median of each group. *, p <0.05.

High levels of granulysin coexisted with relatively large number of CD8$^+$ T cells, but not proportional to *i*NKT, Vγ9$^+$Vδ2$^+$T, CD4$^+$ T cells and NK cells in patients with HIV/TB coinfection in this study. In fact, the decreased CD4$^+$ T cells in patients with HIV/TB coinfection were previously shown to be associated with failure of granuloma formation to contain *Mtb* infection, thereby causing mycobacterial dissemination [28]. Furthermore, activation of HIV-1 by *Mtb* components may be particularly important in viral expansion at the times when *Mtb* growth becomes exponential, and TB overwhelms host defenses [29]. Compared to HIV/TB coinfection, the significantly decreased *i*NKT, Vγ9$^+$Vδ2$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells and increased NK cells in patients with TB before anti-TB therapy indicated the possibility that granulysin may be compromised by T cell subset with significantly correlated with CD8$^+$ T cells. Obviously, increased NK cells were correlated with decreased granulysin, suggesting that it may not be released from NK cells, or granulysin inside the cells does not degranulated later upon activation. However, it could not be assured that (i) how much granulysin contained intracellularly and released to extracellular space since only free granulysin in circulation was measured in this study, (ii) how frequent expression of granulysin was induced in accumulated immune cells including CD8$^+$ T cells at the TB-affected

sites, particularly in patients with HIV/TB coinfection, (iii) the exact duration of being infected with *Mtb* until development to active disease with sputum AFB positive, (iv) how much granulysin was released in latent and early stage of active TB, and (v) varied granulysin levels may depend upon the nutrition and health status in individuals. Similarly, low perforin in TB were seen. The persistence of clinical disease was evidenced to be associated with deficient expression of perforin and granulysin at the local site of TB infection [18]. Although significant infiltration of $CD4^+$ and $CD8^+$ T cells was evidenced in TB lesions in patients with persistent inflammation [18], however, the levels of either perforin or granulysin remained low in TB lesions including severely impaired expression of these cytolytic effector molecules inside the distinct granules [18]. In the present study, either NK cells or $CD8^+$ T cells were significantly correlated with the elevation of perforin in patients with HIV/TB coinfection but not in TB, indicating their potential functions in HIV/TB coinfection, as previously shown in individuals chronically infected with HIV-1 who have increased extracellular perforin levels compared with uninfected individuals, while the impaired functional activity of CTLs and NK cells during HIV-1 infection has been attributed to the decreased intracellular levels of perforin and granzyme B [30]. It is suggested that the induction of perforin has a distinct pathway from that of granulysin in HIV/TB coinfection in this study. However, in TB, no differences in granzyme B levels were found which is supported by similar results in slow or fast TB responders upon TB treatment at any time points and healthy individuals with PPD positive [31].

In contrast to granulysin, perforin and granzyme-B, the elevated circulating IFN-γ seen in patients with both HIV/TB coinfection and TB before anti-TB therapy which decreased after completion of therapy inferred a role of IFN-γ in effective immunity against *Mtb* infection. The results were similar to the previous report on significantly higher plasma IFN-γ levels in patients with active pulmonary TB than healthy individuals which decreased after treatment, suggesting that the levels may result from local production and spill-over of IFN-γ from activated lymphocytes sequestered at the site of *Mtb* infection [22, 24, 32]. In human and mouse models, IFN-γ is evidenced to be normally synthesized from $CD4^+$ T cells activated upon recognition of *Mtb* antigen on antigen presenting cells [22] as well as by *Mtb* antigens specific $CD8^+$ T cells [33]. Although IFN-γ producing $CD4^+$ T cells of Th1 type is of major importance, however, other T cells notably $CD8^+$ T cells and perhaps γδ T cells or CD1-restricted T cells participate in immune function as well [34]. However, increased $CD4^+$ and decreased $CD8^+$ T cells in TB in this study conversed to HIV/TB coinfection. Elevated IFN-γ levels in HIV/TB coinfection might be possibly due to the persistence of immune activation and chronically HIV associated TB. In addition, *Mtb* infection may support the HIV-1 replication and dissemination through dysregulation of host cytokines, chemokines and their receptors [29]. HIV/TB coinfection could inhibit cell mediated responses to *Mtb* through interruption of IL-2 signaling as well [35]. The deleterious effects of HIV infection in $CD4^+$ T cells impair immune function as resulting in a failure to contain mycobacterial infection and restrict the replication of the microbe [36].

In this study, IFN-γ had a trend to increase along with NK cells in patients with both HIV/TB coinfection and TB, suggesting the possible production of IFN-γ from NK cells during initiation against *Mtb* infection which high NK cells were shown. It is also possible as recently shown that NK cells secrete IFN-γ which stimulates monocytes to produce IL-15 and IL-18, which in turn facilitates expansion of $CD8^+$ T cells producing IFN-γ in response to *Mtb*-infected monocytes [37]. Since NK cells produce IFN-γ in early *Mtb* infection [38], therefore, this pathway is likely to be important in facilitating expansion of $CD8^+$ T cells during immune response to *Mtb in vivo* [37].

In conclusion, the alteration of circulating granulysin, perforin and IFN-γ has potential function in host immune response in TB and HIV/TB coinfection. Circulating granulysin and perforin levels in TB were lower than healthy controls, whereas the granulysin levels in HIV/TB coinfection were much higher than in any other disease groups. Increased granulysin and decreased IFN-γ levels in HIV/TB coinfection and TB after completion of anti-TB therapy were noted. Slightly high perforin levels in HIV/TB coinfection indicated the immune activation in TB associated with HIV infection. Three distinct isoforms with ~17kDa, 15kDa and 9kDa of granulysin were recognized in plasma of HIV/TB coinfection. The number of $CD8^+$ T cells kept high but NK cells and other possible cellular sources of granulysin were decreased in HIV/TB coinfection, which in contrast to what seen in TB in which low $CD8^+$ T cells but high NK cells were found, suggesting their different sources of granulysin, which in turn, play a crucial role in host defense against tuberculosis and in association with HIV infection. This is the first demonstration so far of granulysin in HIV/TB coinfection.

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Abbreviations

CFP: culture filtrate protein; CTL: cytotoxic T lymphocyte; ELISA: enzyme-linked immunosorbent assay; ESAT: early secreted antigenic target; HAART: highly active antiretroviral therapy; HIV: human immunodeficiency virus; IFN-γ: interferon gamma; IL: interleukin; *i*NKT: invariant NKT; MDR: multi-drugs resistance; *Mtb*: *Mycobacterium tuberculosis*; NK: natural killer; PBMCs: peripheral blood mononuclear cells; TB: tuberculosis; Th1: T-helper type 1; TLR: toll-like receptor; TNF: tumor necrosis factor; XDR: extensively drugs resistance

Competing Interests

The authors have declared that no competing interest exists.

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Simple Multiplex PCR Assay for Identification of Beijing Family *Mycobacterium tuberculosis* Isolates with a Lineage-Specific Mutation in *Rv0679c*

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The Beijing genotype of *Mycobacterium tuberculosis* is known to be a worldwide epidemic clade. It is suggested to be a possibly resistant clone against BCG vaccination and is also suggested to be highly pathogenic and prone to becoming drug resistant. Thus, monitoring the prevalence of this lineage seems to be important for the proper control of tuberculosis. The Rv0679c protein of *M. tuberculosis* has been predicted to be one of the outer membrane proteins and is suggested to contribute to host cell invasion. Here, we conducted a sequence analysis of the *Rv0679c* gene using clinical isolates and found that a single nucleotide polymorphism, C to G at position 426, can be observed only in the isolates that are identified as members of the Beijing genotype family. Here, we developed a simple multiplex PCR assay to detect this point mutation and applied it to 619 clinical isolates. The method successfully distinguished Beijing lineage clones from non-Beijing strains with 100% accuracy. This simple, quick, and cost-effective multiplex PCR assay can be used for a survey or for monitoring the prevalence of Beijing genotype *M. tuberculosis* strains.

The *Mycobacterium tuberculosis* Beijing genotype, first identified by van Soolingen et al. (1), is known to be a worldwide epidemic clade (2–4). Its possible resistance to BCG vaccination, in addition to its tendency to have a multidrug-resistant (MDR) phenotype, might give a selective advantage to the wide geographic distribution of the Beijing genotype strains (3, 5–7). Although some of the Beijing genotype strains show hypervirulence in animal infection models (7–9), neither the virulence factor nor the phenotypically specific factor of this lineage has been elucidated. The origin of the Beijing lineage is thought to be east Asia, where the prevalence of this clade is from around 40% to >90% (1, 3, 4, 10–13). However, in some other global areas, i.e., countries in the former Soviet Union and South Africa, the prevalence of the Beijing lineage has increased markedly in a short period, and some increases were suggested to be related to MDR (4, 11, 14). In those areas, higher clonality of the circulating strains was suggested, and most were categorized as being in the modern or typical Beijing clade, which is defined as a strain having one or two IS*6110* insertions in the noise transfer function (NTF) chromosomal region (11, 15). On the other hand, a higher variety of strains can be observed in east Asian countries. Especially in Japan and Korea, the majority of the strains belong to another cluster called the ancient or atypical Beijing clade (12, 16). Details regarding the higher pathogenicity of the Beijing lineage are controversial. Some studies have suggested that the modern Beijing clade is more prone to be pathogenic, tends to be drug resistant, and is likely able to escape from BCG vaccination (4, 8, 11, 14); however, some of the ancient Beijing clones were also shown to have higher pathogenicity (17) or a tendency toward acquiring drug resistance (16).

Since Beijing lineage prevalence has a great impact on the tuberculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS*6110* restriction fragment length polymorphism (RFLP) signatures. Soon after, these strains were shown to have a specific spoligotype pattern lacking spacer numbers 1 to 34, and this has been proposed as the definition of the clade (18, 19), since IS*6110* RFLP genotyping is time-consuming, and comparing results between laboratories is difficult. The deletion of spacers observed in the Beijing spoligotype is caused by the insertion of IS*6110* in the direct repeat (DR) region (18). Since this typical spoligotype pattern has become a specific marker of the Beijing genotype, some PCR methods to detect this specific deletion, named region of difference 207 (RD207), have been developed (20–22). In addition to RD207, another deleted region named RD105 was also shown to be a good marker for discrimination of the Beijing genotype, although this deletion is common for all the east Asian lineages, including the non-Beijing strains (10, 23); however, most of these published detection methods require expensive real-time PCR equipment and high-cost reagents (24). The conventional PCR assay targeting RD207 still seems to be at a disadvantage, since it relies on an unstable inser-

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tion sequence that is likely to be a target of homologous recombination.

Instead of unstable repetitive structures, single nucleotide polymorphisms (SNPs) were recently considered to be a robust target for defining the accurate position of a strain on the phylogenetic tree, since horizontal gene transfer or gene recombination between different strains is rare in the *M. tuberculosis* complex (MTC) (12, 24, 25). Filliol et al. (26) drew phylogenetic trees of the MTC using several typing methods and showed that the dendrogram drawn with SNPs most accurately reflected the true evolution of the MTC. Some of those SNPs are suggested to be specific to the Beijing or east Asian lineages. In a search for membrane proteins that are suitable for vaccine antigens and/or are targets for the specific detection of the MTC, we found a candidate protein encoded by the *Rv0679c* gene. This protein was expressed on the cell surface as a lipoarabinomannan-associated protein (27, 28), and the coding sequence has an SNP that seems to be specific to the Beijing clade. In this study, we confirmed the lineage specificity of this SNP and developed a simple and low-cost multiplex PCR assay to distinguish the Beijing lineage strains.

MATERIALS AND METHODS

Preparation of genomic DNA from *M. tuberculosis* isolates. *M. tuberculosis* was isolated from the sputa or other clinical specimens of patients by conventional procedures using *N*-acetyl-L-cysteine (NALC)–NaOH. A total of 619 isolates obtained in Japan ($n = 145$), Bangladesh ($n = 122$), Nepal ($n = 110$), Myanmar ($n = 198$), and China (Heilongjiang Province, $n = 44$) were used in this study. Some of these isolates were the same as those in previous studies, and the details are described elsewhere (13, 29–31). Colonies grown on egg-based medium (either Ogawa or Löwenstein-Jensen medium) were resuspended in distilled water and boiled for 20 min, and the supernatant was used in the Bangladeshi and Myanmar samples. In the Japanese and Nepalese samples, colonies were suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (Tris-EDTA [TE] buffer [pH 8]), and 0.5 ml chloroform; 0.5 g glass beads of 0.17-mm diameter was added; and they were disrupted with a bead beater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation at 10,000 × *g* for 5 min, DNA in the supernatant was precipitated by ethanol, and the precipitated genomic DNA was resuspended in TE buffer for further use. In China, bacteria grown in a BACTEC *Mycobacterium* growth indicator tube (MGIT) (Becton, Dickinson and Company, Franklin Lakes, NJ) were used, and DNA was extracted by lysozymes and the phenol-chloroform method (13). All the DNA samples extracted in each country were brought to Japan, and the following steps were carried out in the Hokkaido University Research Center for Zoonosis Control. To determine the specificity of the method, DNAs extracted from five reference MTC strains (i.e., *M. tuberculosis* H37Rv, *Mycobacterium africanum* ATCC 25420, *Mycobacterium orygis* Z0001, *Mycobacterium microti* TC 89, and *Mycobacterium bovis* BCG Tokyo 172) and 30 nontuberculous mycobacterial (NTM) species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, were used.

Gene sequencing and comparison. A subset of 197 *M. tuberculosis* samples, 68 from Japan, 92 from Bangladesh, and 37 from Nepal, were chosen from the total 619 clinical isolates, and the *Rv0679c* gene fragment was amplified by PCR. The PCR mixture contained GoTaq PCR buffer (Promega Co., Madison, WI), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.3 μM each primers og0001 and og0002 (Table 1), 0.5 M betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. Amplification was carried out by applying 35 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, polymerase reaction mixture at 72°C for 40 s, and a final extension at 72°C for 5 min. The amplified DNA fragment was subjected to sequence analysis with BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA) reagents by a sequencer, the 3130 genetic analyzer (Life Technologies Co.), according to the manufacturer's protocol. The *Rv0679c* sequence was also compared with those of 80 whole-genome sequenced MTC strains registered in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) or TB (http://genome.tbdb.org/annotation/genome/tbdb/MultiHome.html) (32) databases by the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Genotyping. The spoligotype of *M. tuberculosis* clinical isolates was determined as described previously (33). Briefly, the DR region was amplified with a primer pair, and the PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes against the international spoligotyping database (SpolDB4) (3).

The detection of an RD105 deletion was performed by multiplex PCR in Beijing clones and by conventional PCR in east Asian strains other than those of the Beijing type, since the deletion pattern is different between those two groups (10). The reaction mixture consisted of GoTaq PCR buffer (Promega), 0.2 mM each dNTP, 0.3 μM (each) two or three primers (Table 1), 0.5 M betaine, 1 μl extracted DNA sample, and 0.5 units of GoTaq polymerase. The target was amplified by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. RD207 deletion was detected by two PCR assays described by Warren et al. (22), and TbD1 was detected by PCR using the Huard et al. (25) protocol (Table 1). The amplified DNA fragment was subjected to agarose gel electrophoresis with ethidium bromide (EtBr) to see the size of the band under a UV transilluminator.

The multilocus sequence type (MLST) was determined with 9 SNPs, which were described by Filliol et al. (26) and were selected for Beijing subtyping by Iwamoto et al. (16). Each locus was amplified with a primer pair (Table 1), and the product was subjected to sequencing. SNPs were detected by comparing the sequences with those of H37Rv (34). The sequence type (ST) was identified according to Filliol et al. (26).

Beijing lineage identification by multiplex PCR. Multiplex PCR for the identification of the Beijing lineage was performed under the following conditions. The PCR mixture, in a final volume of 15 μl, contained 1× PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5 μl dNTP solution mix (10 mM each dNTP; New England BioLabs, Inc., Ipswich, MA), 0.5 μl each of Fw and R1 primers, 0.2 μl R2 primer (primer solutions in 10 μM; Table 1), 1.5 μl of 5 M betaine, 0.45 μl of 25 mM $MgCl_2$ (to make a final Mg concentration of 2.25 mM), 1 ng of sample DNA, and 0.5 units of TaKaRa Hot Start *Taq* polymerase (TaKaRa). Amplification was carried out with the first denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 10 s, extension at 72°C for 15 s, and the final extension at 72°C for 3 min. The amplicon was subjected to electrophoresis in a 2% agarose gel that included EtBr. DNA samples extracted from the isolate BCG Tokyo 172 and a well-characterized clinical isolate (Beijing OM-9) were used as controls for the non-Beijing and Beijing banding patterns, respectively. Sensitivity was determined with serially diluted genomic DNA obtained from these BCG and Beijing control strains. A specificity study was performed with genomic DNA samples (2 ng/μl each) from the MTC and NTM strains described above.

RESULTS

Spoligotyping and MLST. A total of 619 clinical isolates were subjected to spoligotyping, and 393 were identified as being in the Beijing lineage and 226 as a non-Beijing group (Table 2). The non-Beijing group consisted of a variety of strains belonging to the following lineages: east African-Indian (EAI), central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, S, T, X, and non-Beijing east Asian (3). Ninety-four of the Beijing isolates were subjected to MLST analysis and were subtyped into 8 sequence-type classes, namely, ST26, ST3, STK, ST25, ST19, ST10, ST22, and ST8, which are listed in evolutional order from ancient to modern Beijing types (16, 26).

TABLE 1 Primers used in the study

Target	Primer name	Nucleotide sequence	Purpose	Reference
Rv0679c	og0001	CCGGGAACTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1)[a]	TCGGTCACCGTTTTTGTAGGTGACCGTC	Multiplex PCR for Beijing type identification	This study
	ON-1127 (R2)	AGCAACCTCGCAATCTGACC	Multiplex PCR for Beijing type identification	This study
RD105	RD105-F (−239~−218)	GGAAAGCAACATACACACCACG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-R	AGGCCGCATAGTCACGGTCG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-M (+304~323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination[b]	This study
	RD105EA-F (−80~−60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination[c]	This study
	RD105EA-R (61~42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination[c]	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTCTACTCTGCGCTTTG	PCR for Beijing type identification (set 1)	22
	RD207-2F (Warren)	ACCGAGCTGATCAAACCCG	PCR for Beijing type identification (set 2)	22
	RD207-2R (Warren)	ATGGCACGGCCGACCTGAATGAACC	PCR for Beijing type identification (set 2)	22
TbD1	TbD1F	CGTTCAACCCCAAACAGGTA	PCR for ancestral *M. tuberculosis* determination	25
	TbD1R	AATCGAACTCGTGGAACACC	PCR for ancestral *M. tuberculosis* determination	25
797736[d]	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164[d]	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTCGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596[d]	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067[d]	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015[d]	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133[d]	Beijing ST-7F	TCTTGCGACCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614[d]	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579[d]	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829[d]	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

[a] Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5′ end are not complementary sequences.
[b] This assay was used for Beijing genotype strains.
[c] This assay was used for non-Beijing genotype strains.
[d] This SNP nucleotide position on the *H37Rv* genome is according to references 26 and 34.

Sequence analysis of the *Rv0679c* gene of *M. tuberculosis* isolates. Nucleotide sequences of the full-length *Rv0679c* gene obtained from 197 clinical *M. tuberculosis* isolates collected in Japan, Bangladesh, and Nepal were compared with the *Rv0679c* sequence in *M. tuberculosis* H37Rv (34). Only a single nucleotide difference of cytosine to guanine at position 426, which leads to an amino acid change at codon 142 from Asn (AAC) to Lys (AAG), was detected in 87 isolates, all of which were identified as being in the Beijing lineage by spoligotyping and, supportively, by RD207 PCR (22) (data not shown). One Bangladeshi isolate showed a mixed peak of C and G at position 426 and was revealed as a mixed culture of Beijing and another strain by RD105 and RD207 detection PCR (Table 2). None of the non-Beijing isolates had the mutation, and vice versa. In public databases, 14 strains reported from several countries were revealed to have this mutation, and all were confirmed as being in the Beijing lineage by checking for the RD207 deletion *in silico* (18). None of the other 66 MTC strains, which were determined to be non-Beijing, had this mutation. The 498-bp *Rv0679c* sequence was well conserved among the MTC strains, and the following three strains in the database showed alterations: *M. tuberculosis* strains C and T17 and *Mycobacterium canettii* CIPT 140010059.

TABLE 2 *Rv0679c* multiplex PCR results compared with other typing results in 619 *M. tuberculosis* clinical isolates

Isolate origin	Spoligotype family[a]	RD207, RD105, or other typing methods[b]	Sequence type[c]	*Rv0679c* M-PCR type[d]	No. of isolates
Beijing or Beijing-like					393
Japan	Beijing	ND	26	Beijing	10
	Beijing	ND	3	Beijing	24
	Beijing	ND	STK	Beijing	13
	Beijing-like	RD207$^+$	STK	Beijing	1
	Beijing	ND	25	Beijing	3
	Beijing	ND	19	Beijing	9
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	4
	Beijing	ND	ND	Beijing	23
Bangladesh	Beijing	ND	26	Beijing	3
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	2
	Beijing	ND	8	Beijing	1
	Beijing	ND	ND	Beijing	29
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
Nepal	Beijing	ND	ND	Beijing	64
Myanmar	Beijing	ND	ND	Beijing	141
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	Beijing	ND	ND	Beijing	40
Non-Beijing or undesignated/new[e]					216
Japan	Undesignated/new[e]	RD105$^+$, RD207$^-$	ND	Non-Beijing	29
	Others[f]	ND	ND	Non-Beijing	16
Bangladesh	—[g]	ND	ND	Non-Beijing	73
Nepal	—[h]	ND	ND	Non-Beijing	45
Myanmar	—[i]	ND	ND	Non-Beijing	51
China (Heilongjiang)	Undesignated/new	ND	ND	Non-Beijing	2
Mixed clone samples					6
Bangladesh	Undesignated/new	Mixed peak in sequence[j] RD105$^+$, RD207$^+$	ND	Beijing	1
Myanmar	Undesignated/new	RD105$^+$, RD207$^+$	ND	Beijing	2
	EAI2_NTB	RD105$^+$	ND	Beijing	1
	EAI5	RD105$^+$	ND	Beijing	1
China (Heilongjiang)	Undesignated/new	RD105$^+$	ND	Beijing	1
New spoligotype lacking spacers 1–34[k]					4
Japan	New	RD105$^+$, RD207$^+$[k]	ND	Beijing	1
Nepal	New	RD105$^-$, TbD1$^+$[k]	ND	Non-Beijing	1
Myanmar	New	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	New	RD105$^+$, RD207$^+$	ND	Beijing	1

[a] Spoligotype labeling is according to SpolDB4 (3).
[b] A positive superscript indicates that a deletion was detected; a minus superscript indicates that the RD was not deleted or the region was intact. ND, not determined.
[c] Sequence type is according to reference 26.
[d] M-PCR, multiplex PCR.
[e] East Asian lineage.
[f] Including the clades LAM1, LAM9, T1, T2, T3, T3-Osaka, and new (other than the east Asian lineage).
[g] Including the clades EAI1_SOM, EAI2-MANILA, EAI3_IND, EAI5, EAI6_BGD1, EAI7_BGD2, EAI unidentified, CAS, CAS1-DHLHI, CAS2, LAM9, T1, T4, H1, H3, X1, X2, and undesignated/new.
[h] Including the clades EAI3_IND, EAI5, CAS, CAS1-DHLHI, LAM1, LAM5, T1, T2, T3, H3, S, and undesignated/new.
[i] Including the clades EAI2_MANILA, EAI2_NTB, EAI5, EAI6_BGD1, EAI7_BGD2, CAS1-DHLHI, LAM9, T1, T3, X2, S, and undesignated/new.
[j] Overlapped peak of C and G was observed at nucleic acid position 426.
[k] Details are described in Table 3.

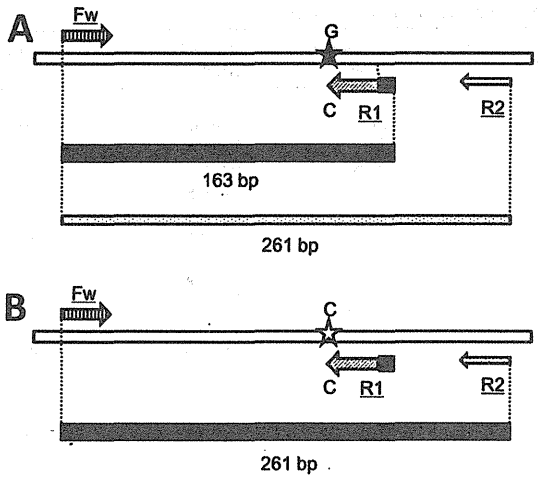


FIG 1 PCR primers and products of *Rv0679c*-targeting multiplex PCR for Beijing lineage discrimination. (A) In the Beijing sample, the 163-bp product is amplified more dominantly than is the 261-bp product. (B) In the non-Beijing sample, 163-bp product is not amplified because of the mismatch of the 3′ end of R1. Fw, forward primer; R1, reverse primer 1 (Beijing lineage specific); R2, reverse primer 2. Two-base noncomplement nucleotides at the 5′ end are shown by black squares.

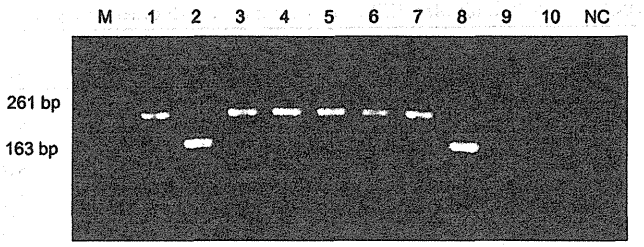


FIG 2 Electrophoresis results of the multiplex PCR products. Lane M, 50-bp ladder DNA size marker; lane 1, *M. bovis* BCG Tokyo 172 (non-Beijing lineage control) strain; lane 2, *M. tuberculosis* OM-9 strain (Beijing lineage control); lane 3, *M. tuberculosis* H37Rv; lane 4, *M. africanum* ATCC 25420; lanes 5–8, *M. tuberculosis* clinical isolates (lane 5, non-Beijing east Asian; lane 6, EAI; lane 7, LAM9; lane 8, Beijing); lane 9, *M. avium* strain JATA51-1; lane 10, *M. kansasii* JATA21-1; lane NC, negative control.

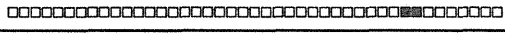
In strain C, the C185T SNP was observed, and in T17, a cytosine was inserted at position 92. In *M. canettii* CIPT 140010059, two SNPs and a codon insertion, ACC at position 154, were observed.

Beijing lineage identification by multiplex PCR. Multiplex PCR was developed targeting the Beijing-specific SNP on *Rv0679c*, employing a primer with the mutated nucleic acid at the 3′ end of the sequence (primer R1; Fig. 1 and Table 1); the optimal reaction conditions were determined as described in Materials and Methods. With this system, a bright band of 163 bp was observed as an amplified product of the primers Fw and R1 in the Beijing genotype samples (Fig. 1A and 2). An additional band of 261 bp, which is the product of primers Fw and R2, can be seen depending on the conditions, although it is always significantly thinner than the 163-bp band because of the low R2-primer concentration (see Materials and Methods). In contrast, only the 261-bp band is observed in a non-Beijing genotype sample (Fig. 1B and 2). Since the sequences of the primers are specific to the MTC, no amplification occurs in the absence of MTC genomic DNA (Fig. 2, data for *M. avium* and *M. kansasii*). A total of 619 clinical isolates obtained in the five Asian countries of Japan, Bangladesh, Nepal, Myanmar, and China were subjected to this Beijing lineage-identifying multiplex PCR, and the results were compared with their spoligotypes. All the isolates determined as having a Beijing or Beijing-like genotype by the SpolDB4 ($n = 393$) were determined to be in the Beijing lineage by the multiplex PCR (Table 2). On the other hand, no samples that included only non-Beijing genotype DNA ($n = 216$) were identified as being in the Beijing lineage. Twenty-nine non-Beijing east Asian lineage strains, which were suggested by a characteristic spoligotype having spacer 34 and were defined by RD105 detection, were determined to be non-Beijing by the multiplex PCR. Six isolates that showed a discrepancy between their spoligotype and the multiplex PCR result were further determined by RD207 or RD105 detection PCR and were revealed to be a mixture of Beijing and other subtype strains (mixed clone samples, Table 2). Four samples from different countries had confusing spoligotypes that lacked spacers 1 to 34 and additionally lacked some of the spacers from 35 to 43. These samples could also be identified correctly (Tables 2 and 3). The minimum detection limits were 100 and 1,000 cells per reaction in the Beijing genotype and BCG strains, respectively (data not shown).

DISCUSSION

In this study, we demonstrated that the SNP of C to G at position 426 in the *Rv0679c* gene is specific to the Beijing genotype strains. We developed a new multiplex PCR using this SNP to identify Beijing lineage isolates. This PCR assay successfully distinguished Beijing genotype strains from others, including the non-Beijing east Asian strains, with 100% accuracy. The Beijing lineage genotype is usually identified by spoligotyping, specific patterns of IS*6110* RFLP, or the detection of RD207, which is led by an insertion of IS*6110* in the DR region. However, spoligotyping is well known to show gene conversions, and strains having no genetic relationship sometimes show the same spoligotype (3, 26). Fenner et al. (35) reported pseudo-Beijing strains that had a typical Beijing spoligotype even though they actually belonged to the CAS family. This type of confusion seems to occur especially in areas that have a higher prevalence of principal genetic group 1 (PGG1) lineages, including the EAI, CAS, and east Asian lineages, since PGG1 strains usually possess spacers 35 and 36, which are lacking in PGG2 and PGG3 strains (3, 36). In other areas, mixed infections of more than two strains sometimes disrupt correct spoligotyping by showing mixed spacer patterns. The Manu1-SIT100 and Manu2-SIT54 types, which lack the spacers 34 or 33 and 34, respectively, are known to be producible by the mixture of Beijing family and T1 strains (3, 37). In this study, we found that some samples showed discrepant results between *Rv0679c* multiplex PCR and spoligotyping that determined a strain to be of the Beijing genotype by multiplex PCR, despite having another spoligotype. Using RD105 and RD207 detection methods, all of these samples were confirmed to be a mixture of Beijing and another strain. This type of mixed culture is sometimes observed in countries with a higher TB burden, where a coinfection of more than two strains is not rare (22). Some of the spoligopatterns of those samples showed faint positive spacers, suggesting the mixed presence of other strains. Even clear and correct spoligotypes can sometimes lead to misjudgments. In the current study, some samples showed only one to several spacers to be positive in the Beijing spacer area,

TABLE 3 Typing result comparison in clinical isolates having confusing spoligotype patterns

Sample (identification)	Spoligotype pattern	Spoligotype family[a]	*Rv0679c* M-PCR type	Detection type				Final typing result
				RD207 set 1[b]	RD207 set 2[b]	RD105	TbD1	
Japan (O-05-44)		New	Beijing	−	+[c]	+	−	Beijing
Nepal (no. 51)		New	Non-Beijing	−	−	−	+	Ancestral[d]
Myanmar (no. 95)		New	Beijing	+	+	+	ND	Beijing
China (2460)		New[e]	Beijing	+	+	+	ND	Beijing

[a] These patterns were not found in the SpolDB4 list.
[b] PCR sets 1 and 2 in reference 22.
[c] A faint correctly sized band and an additional band of a different size were observed.
[d] Ancestral type of *M. tuberculosis* strain possessing TbD1 region (25).
[e] The spoligotype pattern of this sample has been reported in reference 13.

namely, from spacers 35 to 43. Most were identified as being of the Beijing genotype by multiplex PCR, while one was judged to be a non-Beijing strain. All Beijing genotype-positive results were confirmed by RD105 and RD207 PCRs, and the non-Beijing isolate was revealed as an "ancestral type," which involves EAI but not the Beijing lineage, by TbD1 detection (Table 3) (25). These examples support the high specificity and applicability of this SNP-targeting PCR. The disadvantages of IS*6110* RFLP and RD207 detection have already been described above. RD207-detection PCR did not work as expected in the sample that lost spacers 1 to 42 (Japan O-05-44; Table 3), suggesting that some additional reconstruction had occurred at the IS*6110* insertion site of the DR region. SNPs in MTC genomes can provide robust lineage information, whereas repetitive elements, such as direct repeats in the DR region, the mycobacterial interspersed repetitive unit (MIRU) tandem repeats (38), or IS*6110*, are prone to alteration. One hundred percent concordance of the PCR results with the genetically confirmed Beijing type is not surprising because of the rigidity of the SNPs in the MTC (25, 26). Of the 393 Beijing family isolates, 94 were subtyped by MLST and consisted of 8 STs covering a wide range of the Beijing family, from ancient to modern types (Table 2). This suggested that a specific mutation in *Rv0679c* seemed to have occurred in the Beijing lineage at the same time as the RD207 deletion event.

Rv0679c is an MTC-specific gene, as shown by Cifuentes et al. (27), and no significantly similar sequence was detected by an NCBI BLASTn search in the GenBank database. Thus, this multiplex PCR assay can be used for the identification of the MTC, as well as for the differentiation of Beijing and non-Beijing lineages (Fig. 2). The Beijing mutation detection primer (R1; Fig. 1 and Table 1) was designed to have two additional noncomplement bases at the 5′ end to block the second amplification by the PCR product that produces the 261-bp fragment with an outer R2 primer. Additionally, the higher concentration and melting temperature of the R1 primer compared to those of the outer R2 primer increase the Fw-R1 product more than the Fw-R2 product. With these techniques, the Beijing band (163 bp) can be shown to be significantly brighter than the non-Beijing band (261 bp) when the sample is derived from Beijing lineage *M. tuberculosis* strains (Fig. 2). The relatively higher annealing temperature of 66°C gave good contrast of those two bands and prevented nonspecific amplifications. Modified *Taq* or other polymerases that have 3′-to-5′ exonuclease activity should be avoided, since those enzymes can trim the mutated nucleotide at the R1 primer end. It is recommended to check the PCR conditions using positive controls for Beijing and non-Beijing types (i.e., BCG) every time (Fig. 2). The detection limit of 100 to 1,000 copies per reaction might be relatively high; however, it can be improved by about 10 times by increasing the PCR cycle number to 40, although the necessity of identifying the MTC lineage in direct clinical specimens seems to be low.

In papers featuring SNPs as epidemiological markers, synonymous mutations are usually selected to avoid the effect of evolutional pressure (26). However, both SNPs for the differentiation of PGG1, PGG2, and PGG3 were nonsynonymous mutations in *katG* and *gyrA* (36), and so far, they have provided robust differentiation results. In the MTC, nonsynonymous mutations on functional genes can be observed in a relatively higher frequency than in other bacteria because of extremely reduced purifying selection pressure (39). Thus, nonsynonymous mutations can be preserved unless they are significantly disadvantageous. Indeed, 100% of the Beijing family strains in the current study could be identified with this nonsynonymous mutation, suggesting that it at least has no adverse effect on those strains. The function of the Rv0679c protein is still unclear, although its expression on the cell surface has been confirmed (27, 28). Cifuentes et al. (27) reported that the surface-localized Rv0679c protein contributed to the *M. tuberculosis* invasion of host cells and proposed the protein as a vaccine candidate. The substituted amino acid at position 142 was located in the C-terminus region of the protein, which was included in the "high-activity binding peptide" to target cells (27). Thus, this highly conserved nonsynonymous SNP, which results in an amino acid substitution with different characteristics (Asn → Lys), might have some biological meaning in explaining Beijing lineage pathogenicity. Since BCG vaccine strains, as well as other non-Beijing strains, have Rv0679c-Asn142, this substitution might affect the antigenicity of the Beijing bacterial surface and might contribute to the possible evasion of BCG-derived immunity. Further investigation of the association of the Rv0679c Asn142Lys substitution with Beijing strain outer membrane characteristics and antigenicity is ongoing.

In conclusion, a simple, robust, and low-cost multiplex PCR assay for the detection of Beijing lineage *M. tuberculosis* strains was successfully developed using a Beijing-specific SNP on *Rv0679c*. This PCR assay can be used in local laboratories to monitor the prevalence of the Beijing genotype, and this is strongly recommended to control this possibly highly pathogenic and drug resistance-prone sublineage.

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Primary Drug-Resistant Tuberculosis in Hanoi, Viet Nam: Present Status and Risk Factors

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Abstract

Introduction: Resistance of *Mycobacterium tuberculosis* (MTB) to anti-tuberculosis (TB) drugs presents a serious challenge to TB control worldwide. We investigated the status of drug resistance, including multidrug-resistant (MDR) TB, and possible risk factors among newly diagnosed TB patients in Hanoi, the capital of Viet Nam.

Methods: Clinical and epidemiological information was collected from 506 newly diagnosed patients with sputum smear- and culture-positive TB, and 489 (96.6%) MTB isolates were subjected to conventional drug susceptibility testing, spoligotyping, and 15-locus variable numbers of tandem repeats typing. Adjusted odds ratios (aORs) were calculated to analyze the risk factors for primary drug resistance.

Results: Of 489 isolates, 298 (60.9%) were sensitive to all drugs tested. Resistance to isoniazid, rifampicin, streptomycin, ethambutol, and MDR accounted for 28.2%, 4.9%, 28.2%, 2.9%, and 4.5%, respectively. Of 24 isolates with rifampicin resistance, 22 (91.7%) were MDR and also resistant to streptomycin, except one case. Factors associated with isoniazid resistance included living in old urban areas, presence of the Beijing genotype, and clustered strains [aOR = 2.23, 95% confidence interval (CI) 1.15–4.35; 1.91, 1.18–3.10; and 1.69, 1.06–2.69, respectively). The Beijing genotype was also associated with streptomycin resistance (aOR = 2.10, 95% CI 1.29–3.40). Human immunodeficiency virus (HIV) coinfection was associated with rifampicin resistance and MDR (aOR = 5.42, 95% CI 2.07–14.14; 6.23, 2.34–16.58, respectively).

Conclusion: Isoniazid and streptomycin resistance was observed in more than a quarter of TB patients without treatment history in Hanoi. Transmission of isoniazid-resistant TB among younger people should be carefully monitored in urban areas, where Beijing strains and HIV coinfection are prevalent. Choosing an optimal treatment regimen on the basis of the results of drug susceptibility tests and monitoring of treatment adherence would minimize further development of drug resistance strains.

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Introduction

Resistance of *Mycobacterium tuberculosis* (MTB) to anti-tuberculosis (TB) drugs, particularly to isoniazid (INH) and rifampicin (RMP), which results in multidrug-resistant (MDR)-TB, presents a serious challenge in the control of TB worldwide [1,2]. The World Health Organization (WHO) estimates that the prevalence of MDR-TB varies from 0% to 65.1% across the world [1]. Despite progress in disease surveillance, more than 80% of MDR-TB patients are unaware of their disease status, indicating that the transmission status of MDR-TB is mostly unknown in high-TB burden countries [1].

Drug-resistant TB, including MDR-TB, develops as a result of inadequate treatment of an individual who was initially infected

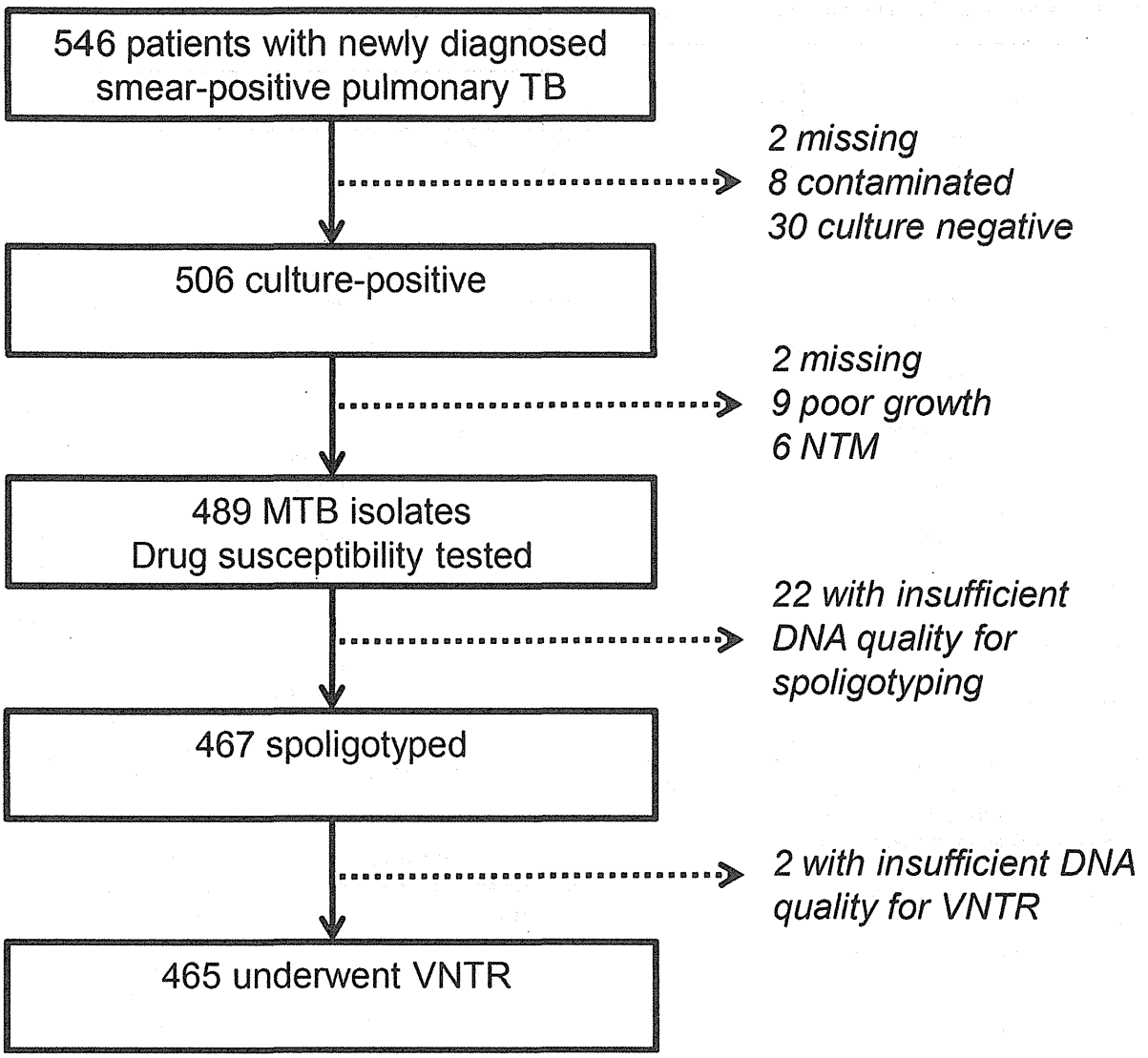

Figure 1. Study flow. TB: tuberculosis; MTB: *Mycobacterium tuberculosis*; NTM: nontuberculous mycobacterium; VNTR: variable numbers of tandem repeats; DNA: deoxyribonucleic acid.
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with a fully or partly sensitive strain or by direct transmission of a drug-resistant strain from one individual to another [3]. Although previous treatment is the strongest risk factor of MDR-TB, other risk factors such as younger age, male gender, and human immunodeficiency virus (HIV) coinfection have also been reported [4-6]. Further analysis may provide information on the dynamics of its transmission and better countermeasures against increasingly drug-resistant TB.

Viet Nam is one of the 22 countries with a high TB burden and is one of the 27 countries with a high MDR-TB burden [1]; the prevalence of any drug resistance and MDR-TB among newly diagnosed cases in a 2006 countrywide survey was 30.7% and 2.7%, respectively [7]. Although drug resistance, including MDR, and potential risk factors have been investigated in some areas [8-10], host-, pathogen-, and environment-related factors, such as patients' HIV status; residential area; and genotypes of the MTB isolates, have not been comprehensively assessed in Viet Nam. We conducted this study to estimate the status of primary anti-TB drug resistance, including MDR, among newly diagnosed TB patients in Hanoi, the capital and second largest city of Viet Nam, and to investigate the role of the above risk factors in resistance to each of the first-line drugs.

Table 1. Characteristics of the study population (n = 489).

		Number	%
Age (median, range)		(38.6,	16.6–85.4)
Gender	Male	386	78.9
	Female	103	21.1
Body mass index	<16	70	14.3
	16–18.4	201	41.1
	18.5–24.9	213	43.6
	≥25	4	0.8
	Not available	1	0.2
Residential area	Suburban	100	20.4
	New urban	228	46.6
	Old urban	161	32.9
Smoking habit	Smoker	189	38.7
	Ex-smoker	134	27.4
	Nonsmoker	165	33.7
	No answer	1	0.2
HIV status	Positive	44	9.0
	Negative	443	90.6
	Not available	2	0.4

HIV: human immunodeficiency virus

Table 2. Patterns of INH, SM, RMP, and EMB resistance (n = 489).

Pattern		Number	%
Sensitive with all drugs		**298**	**60.9**
Any resistance	**Total**	**191**	**39.1**
	INH	138	28.2
	RMP	24	4.9
	SM	138	28.2
	EMB	14	2.9
Monoresistance	**Total**	**101**	**20.7**
	INH	49	10.0
	RMP	2	0.4
	SM	50	10.2
	EMB	0	0.0
Polyresistance, non-MDR	**Total**	**68**	**13.9**
	INH + SM	65	13.3
	INH + EMB	1	0.2
	INH + SM + EMB	1	0.2
	RMP + SM	0	0.0
	RMP + EMB	0	0.0
	RMP + SM + EMB	0	0.0
	SM + EMB	1	0.2
MDR	**Total**	**22**	**4.5**
	INH + RMP	1	0.2
	INH + RMP + EMB	0	0.0
	INH + RMP + SM	10	2.1
	INH + RMP + EMB + SM	11	2.2

INH: isoniazid; RMP: rifampicin; SM: streptomycin; EMB: ethambutol; MDR: multidrug resistance

Materials and Methods

Ethics statement

Written informed consent was obtained from each participant. In the case of minors, the parents provided written informed consent. This study was approved by the ethical committees of the Ministry of Health, Viet Nam, and National Center for Global Health and Medicine, Japan, respectively.

Study sites, recruitment of patients, and sample collection

As part of our prospective study project, we included 7 of the 14 districts in Hanoi as the catchment area, where more than half of new smear-positive TB patients in the city were diagnosed and treated in the area during the study period. Among the districts, two were located in the old city area established before 1954 and had a population density that ranged from 25,000 to 26,000 individuals /km^2 in 2009. As such, they were categorized as "old urban" areas. The remaining five districts were originally regarded as suburban areas. Of these, three were recently upgraded to urban areas on the basis of rapid economic development and had a population density that ranged from 2,800 to 5,300 individuals/km^2, although the migrating population was not counted. We categorized these three areas as "new urban." The two other areas remained "suburban," and their population densities ranged from 1,500 to 2,500 individuals/km^2.

Patients were considered eligible if they were 16 years or older, resided in the abovementioned catchment areas, suffered from smear-positive pulmonary TB without a history of TB treatment, and agreed to participate in this study. Eligible patients who visited the local TB care units were recruited consecutively from July 2007 to March 2009. Information about no previous TB treatment was based on interviews conducted by pre-trained health care staff and medical records kept for registration with the National TB Program in district TB centers.

Before initiating anti-TB treatment, sputum specimens were cultured and subjected to identification of MTB, drug susceptibility tests, and DNA extraction for molecular typing. Blood samples were obtained for HIV testing and complete blood count. Bacterial load estimated in sputum smear was used to assess the severity of the disease.

Identification of MTB and drug susceptibility testing

After undergoing solid cultures on Löwenstein–Jensen media, MTB isolates from sputum specimens were subjected to a niacin test. For drug susceptibility testing, the WHO standard proportional method was used to identify resistance to INH, RMP, streptomycin (SM), and ethambutol (EMB) [11]. The test media contained INH (0.2 μg/mL), RMP (40 μg/mL), SM (4 μg/mL), and EMB (2 μg/mL). Resistance to pyrazinamide (PZA) was tested using a pyrazinamidase assay, in which pyrazinamidase activity was determined using Wayne's method with minor modifications [12]. The H37Rv strain of MTB, which is susceptible to PZA and positive for pyrazinamidase, was used as the positive control. The BCG strain of *M. bovis*, which is resistant to PZA and negative for pyrazinamidase, served as the negative control.

Table 3. Characteristics of MDR-TB patients.

No.	Gender, age	Residential area	HIV	DR pattern	MTB spoligotype	VNTR pattern	Clustered among MDR cases	Clustered among all cases
138	M, 40	Old urban	Neg.	IRS	Beijing	233643446844243	Yes (cluster I)	Yes (cluster I)
294	M, 22	Old urban	Neg.	IRSE	Beijing	233643446844243	Yes (cluster I)	Yes (cluster I)
166	M, 50	Old urban	Neg.	IRS	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
347	F, 18	Suburban	Neg.	IRS	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
356	M, 30	New urban	Neg.	IRSE	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
239	M, 43	New urban	Neg.	IRSE	Unclassified	642245742652124	Yes (cluster III)	Yes (cluster III)
256	M, 34	New urban	Pos.	IRSE	Unclassified	642245742652124	Yes (cluster III)	Yes (cluster III)
48	F, 55	Old urban	Neg.	IRS	Beijing	233753447534443	Yes (cluster IV)	Yes (cluster IV)
449	M, 29	Old urban	Pos.	IRS	Beijing	233753447534443	Yes (cluster IV)	Yes (cluster IV)
205	M, 52	Suburban	Neg.	IRSE	Beijing	233751445854242	No	Yes (cluster V)
474	M, 26	New urban	Neg.	IR	Beijing	223753445854243	No	Yes (cluster VI)
36	M, 44	Old urban	Neg.	IRSE	Beijing	243753N42344335	No	No
69	M, 35	New urban	Neg.	IRS	Beijing	233753446754243	No	No
126	M, 26	New urban	Pos.	IRSE	Beijing	233751545854242	No	No
236	M, 34	Suburban	Pos.	IRS	EAI5	632253742692122	No	No
368	M, 40	New urban	Neg.	IRS	Beijing	232543443844443	No	No
409	M, 30	New urban	Pos.	IRSE	Beijing	233455444832423	No	No
489	M, 44	Old urban	Neg.	IRS	Beijing	223753445864243	No	No
528	M, 55	New urban	Neg.	IRSE	Unclassified	642245442652124	No	No
16	M, 62	Old urban	Neg.	IRSE	N/A	N/A	N/A	N/A
264	M, 36	New urban	Pos.	IRSE	EAI5	N/A	N/A	N/A
333	M, 31	New urban	Pos.	IRS	EAI5	N/A	N/A	N/A

HIV: human immunodeficiency virus; MDR-TB: multidrug-resistant tuberculosis; DR: drug-resistant; VNTR: variable numbers of tandem repeats; M: male; F: female; IR: resistant to isoniazid and rifampicin; IRS: resistant to isoniazid, rifampicin, and streptomycin; IRSE: resistant to isoniazid, rifampicin, streptomycin, and ethambutol; Neg: negative; Pos: positive; MTB: *Mycobacterium tuberculosis*; N (in "VNTR pattern" column): polymerase chain reaction negative; EAI: East African–Indian; N/A: not available.

Molecular genotyping

Spoligotyping was performed to confirm the presence of Beijing strains and to identify sublineages of non-Beijing strains using a spoligotyping kit (Ocimum Biosolutions LLC, Houston, TX, USA), according to the standard protocol [13]. Classification of the spoligotype family was based on the international database, SpolDB4 [14].

We analyzed a single-nucleotide polymorphism at the 3284855 position using real-time polymerase chain reaction to further confirm the presence of Beijing strains [15].

Variable numbers of tandem repeats (VNTR) analysis was conducted for all strains using the international standard 15 mycobacterial interspersed repetitive unit (MIRU)-VNTR proposed by Supply et al. [16], with the exception of DNA samples with ambiguous results. The copy number of each locus of the H37Rv strain was used as to confirm the different definition in VNTR analysis. The copy numbers in MIRUs-4, 10, 16, 26, 31, and 40; ETRs-A and C; and VNTRs-2163b, 4052, 1955, 2401, 4156, 0424, and 3690 were defined as 3-3-2-3-3-1-3-4-5-5-2-2-2-2-5, respectively. We defined each cluster by complete match of the VNTR profile. To confirm the appropriateness of each cluster, spoligotyping patterns were also considered. The clustering rate was calculated as described elsewhere [17].

Statistical analysis

The chi-squared test was used to compare the proportions between drug-sensitive and drug-resistant groups. The logistic regression models were used to evaluate potential risk factors for drug resistance, and adjusted odds ratios (aORs) and 95% confidence intervals (CIs) were calculated. Therein, each drug-resistance pattern was set as an outcome variable, and factors that could affect the pattern were chosen as independent variables. For RMP resistance and MDR, only variables with biological significance and with significant associations in univariate analysis were included in the multivariate models, because the number of outcome variables was limited. Statistical analysis was performed using Stata version 11 (StataCorp, College Station, TX, USA), and $P < 0.05$ was considered to be statistically significant.

Results

Study samples and patient characteristics

In total, 546 newly diagnosed smear-positive pulmonary TB patients were recruited. From 506 culture-positive cases, microbial isolates were collected from 495 patients (97.8%), of which six were infected with nontuberculous mycobacteria. As a result, 489 MTB isolates were tested for drug susceptibility. Because of insufficient quality of the extracted DNA samples, 467 MTB isolates further underwent spoligotyping and 465 underwent VNTR typing (Figure 1). The median age was 38.6

Table 4. Univariate analysis using the logistic regression model of the associations between potential risk factors and drug resistance (n = 489).

		Any drug resistance		INH resistance		SM resistance		RMP resistance		MDR	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Age (in years)	≥45	1.00		1.00		1.00		1.00		1.00	
	<45	**1.85**	**1.26–2.71**	**1.85**	**1.21–2.83**	**1.77**	**1.16–2.69**	2.53	0.93–6.90	2.25	0.82–6.21
Sex	Male	1.00		1.00		1.00		1.00		1.00	
	Female	0.72	0.45–1.14	1.06	0.65–1.71	0.63	0.38–1.06	0.33	0.08–1.42	0.36	0.08–1.58
Smoking*	No	1.00		1.00		1.00		1.00		1.00	
	Yes	**1.69**	**1.14–2.51**	1.28	0.84–1.96	**2.00**	**1.28–3.14**	1.89	0.69–5.18	1.67	0.60–4.64
HIV status	Negative	1.00		1.00		1.00		1.00		1.00	
	Positive	**1.98**	**1.06–3.70**	**2.07**	**1.10–3.89**	**2.30**	**1.22–4.31**	**4.74**	**1.85–12.16**	**5.40**	**2.07–14.07**
Number of lymphocytes (cells/mm³)											
	≥1,000	1.00		1.00		1.00		1.00		1.00	
	<1,000	**1.74**	**1.01–3.01**	1.61	0.91–2.85	1.63	0.92–2.89	1.99	0.71–5.54	2.23	0.79–6.30
Smear**		0.91	0.76–1.11	0.90	0.73–1.11	0.88	0.72–1.09	0.89	0.57–1.37	0.92	0.58–1.44
MTB strain	Non-Beijing	1.00		1.00		1.00		1.00		1.00	
	Beijing	**2.00**	**1.35–2.95**	**2.11**	**1.37–3.26**	**1.95**	**1.26–3.00**	1.68	0.68–4.16	1.84	0.70–4.83
Clustered	No	1.00		1.00		1.00		1.00		1.00	
	Yes	**1.66**	**1.13–2.44**	**2.08**	**1.36–3.20**	1.16	0.77–1.75	1.08	0.45–2.62	1.12	0.44–2.83
BMI	18.5–24.9	1.00		1.00		1.00		1.00		1.00	
	<16	0.85	0.49–1.49	0.72	0.38–1.36	0.95	0.52–1.74	0.69	0.19–2.49	0.82	0.22–3.04
	16–18.4	1.02	0.68–1.51	0.99	0.65–1.51	1.03	0.67–1.58	0.64	0.26–1.57	0.76	0.30–1.93
	≥25	1.54	0.21–11.11	2.44	0.34–17.67	0.85	0.09–8.33	-		-	
Residential area											
	Suburban	1.00		1.00		1.00		1.00		1.00	
	New urban	**1.97**	**1.18–3.29**	1.70	0.96–3.03	1.42	0.82–2.45	1.64	0.45–6.01	1.64	0.45–6.01
	Old urban	**1.98**	**1.15–3.40**	**2.15**	**1.18–3.91**	1.38	0.78–2.46	2.14	0.57–7.98	1.69	0.44–6.53

INH: isoniazid; SM: streptomycin; RMP: rifampicin; MDR: multidrug-resistance; HIV: human immunodeficiency virus; BMI: body mass index; MTB: *Mycobacterium tuberculosis*; OR: odd ratios; 95% CI: 95% confidence interval

* Includes ex-smoking.

** OR per unit change of smear positivity (scanty, 1+, 2+, 3+).

Bold type indicates significant associations.

years (range = 16.6–85.4), the proportion of male patients was 78.9%, and HIV coinfection was observed in 9.0% of the patients (Table 1).

Prevalence and patterns of resistance to INH, SM, RMP, EMB, and PZA

Of the 489 MTB isolates, 60.9% were fully sensitive to INH, SM, RMP, and EMB. INH resistance was observed in 138 isolates (28.2%), which included 49 (10.0%) isolates of INH monoresistance; SM resistance was also observed in 138 isolates (28.2%), which included 50 isolates of SM monoresistance (10.2%), and the rest were mostly the combination of INH and SM resistance (Table 2). Primary resistance to RMP was detected in 24 isolates (4.9%), and 22 isolates were MDR-TB, which accounted for 4.5% of all isolates; most of these were also SM resistant. EMB resistance was not frequent (2.9%). The pyrazinamidase assay showed negative results for 12 isolates (2.5%), indicating resistance to PZA. The proportion of PZA resistance among MDR cases was significantly higher than that in non-MDR cases (13.6%, 95% CI 2.9–34.9 vs. 1.9%, 95% CI 0.9–3.6; P = 0.001; Table not provided).

Distribution of MTB lineages and clusters of drug-resistant isolates

Among 467 MTB isolates spoligotyped, the Beijing genotype was most frequently observed [272 isolates (58.2%)]. The East African-Indian (EAI) lineage ranked as the second most frequently observed genotype [93 isolates (19.9%)], of which 84 isolates showed the EAI5 genotype and 9 showed a Vietnamese genotype (EAI4_VNM) (Table not provided). Among 21 of the 22 MDR-MTB strains available for spoligotyping, 15 (71.4%) were of Beijing genotype, 3 (14.3%) were of EAI genotype, and the remaining 3 (14.3%) showed unclassified non-Beijing genotypes but closely resembled EAI4 or 5, according to the spoligotyping database (Table 3).

Of the 465 isolates, in which both spoligotype and VNTR patterns were available, 257 (55.3%) were clustered strains belonging to 55 clusters, indicating that the clustering rate was 43.4% [(257−55)/465]. The proportion of clustered strains was significantly higher in the group with any drug resistance than

Table 5. Results of multivariate analysis using the logistic regression model on the associations between potential risk factors and drug resistance (n = 489).

Factors		Number (%)	Multivariate	
			aOR	95% CI
Any drug resistance*				
Age (in years)	≥45	58/191 (30.4)	1.00	-
	<45	133/298 (44.6)	**1.72**	**1.11–2.66**
Smoking**	No	51/165 (30.9)	1.00	-
	Yes	139/323 (43.0)	**1.87**	**0.99–3.49**
Residential area	Suburban	27/100 (27.0)	1.00	-
	New urban	96/228 (42.1)	**2.06**	**1.17–3.62**
	Old urban	68/161 (42.2)	**2.14**	**1.17–3.91**
MTB strain	Non-Beijing	57/195 (29.2)	1.00	-
	Beijing	123/272 (45.2)	**1.86**	**1.21–2.87**
INH resistance*				
Residential area	Suburban	19/100 (19.0)	1.00	-
	New urban	65/228 (28.5)	1.60	0.85–3.02
	Old urban	54/161 (33.5)	**2.23**	**1.15–4.35**
MTB strain	Non-Beijing	38/195 (19.5)	1.00	-
	Beijing	92/272 (33.8)	**1.91**	**1.18–3.10**
Clustered	No	41/207 (19.8)	1.00	-
	Yes	87/258 (33.7)	**1.69**	**1.06–2.69**
SM resistance*				
Smoking**	No	32/165 (19.4)	1.00	-
	Yes	105/323 (32.5)	**2.47**	**1.18–5.16**
MTB strain	Non-Beijing	39/195 (20.0)	1.00	-
	Beijing	89/272 (32.7)	**2.10**	**1.29–3.40**
RMP resistance***				
HIV	Negative	17/443 (3.8)	1.00	-
	Positive	7/44 (15.9)	**5.42**	**2.07–14.14**
MTB strain	Non-Beijing	7/195 (3.6)	1.00	-
	Beijing	16/272 (5.9)	1.67	0.67–4.20
MDR***				
HIV	Negative	15/443 (3.4)	1.00	-
	Positive	7/44 (15.9)	**6.23**	**2.34–16.58**
MTB strain	Non-Beijing	6/195 (3.1)	1.00	-
	Beijing	15/272 (5.5)	1.84	0.69–4.90

INH: isoniazid; SM: streptomycin; RMP: rifampicin; MDR: multidrug-resistance; TB: tuberculosis; HIV: Human immunodeficiency virus; aOR: adjusted odd ratios; 95% CI: 95% confidence interval

* Only factors showing significant associations were shown.

** Included ex-smoking.

*** The final model included biologically significant variables (MTB lineage) and variables showing significant associations (HIV status) in univariate analysis.

Bold type indicates significant associations.

in the fully-sensitive group [112/178 (62.9%) vs. 145/287 (50.5%), P = 0.009]. Of the 22 MDR isolates, spoligotype and VNTR patterns of MTB were available in 19. Eleven (57.9%) of them belonged to six clusters, I–VI, as determined by a comparison of genotyping patterns observed in the 465 tested isolates, and clusters II and IV were the first (9.5%) and second (3.4%) largest clusters among them (Table not provided). MDR strains in the largest cluster II were observed in all of the old, new, and suburban areas. The VNTR patterns of the clusters I and II were different only in 2 of the 15 loci tested (Table 3).

Factors associated with drug-resistant TB

The logistic regression models were used to identify factors associated with drug resistance. Factors that were analyzed included gender, age, body mass index (BMI), smoking behavior, the patient's residential area, MTB load in the sputum smear before treatment, HIV status, the number of blood lymphocytes, MTB lineage, and clustered strains. Univariate and multivariate analyses (Tables 4 and 5) revealed that age less than 45 years, living in a new or old urban area, and being infected with Beijing strains were significantly associated with any drug resistance (aOR = 1.72, 95% CI 1.11–2.66; 2.06, 1.17–3.62; 2.14, 1.17–3.91; and 1.86, 1.21–2.87, respectively). However, living in an old urban area and being infected with Beijing strains or clustered strains were significantly associated with INH resistance (aOR = 2.23, 95% CI 1.15–4.35; 1.91, 1.18–3.10; and 1.69, 1.06–2.69, respectively), and being a smoker or infection with the Beijing MTB strain showed significant association with SM resistance (aOR = 2.47, 95% CI 1.18–5.16; 2.10, 1.29–3.40, respectively) (Table 5). Younger age was significantly associated with INH and SM resistance in univariate analysis (OR = 1.85, 95% CI 1.21–2.83; 1.77, 1.16–2.69, respectively) (Table 4), but these associations were not significant in multivariate analysis (aOR = 1.59, 95% CI 0.98–2.58; 1.56, 0.97–2.52, respectively) (Table not provided).

Multivariate analyses revealed that only HIV coinfection was significantly associated with RMP resistance (aOR = 5.42, 95% CI 2.07–14.14) and MDR (aOR = 6.23, 95% CI 2.34–16.58) (Tables 4 and 5).

Discussion

We found that the proportion of drug-resistant cases, including MDR, was considerably high among newly diagnosed smear-positive culture-positive pulmonary TB patients residing in Hanoi city. Depending on the type of drug resistance, the drug resistance-associated risk factors showed a pronounced variation and revealed complicated aspects in a large city. The majority of MDR-TB cases revealed that infection with Beijing strains was predominantly spread in this area, while non-Beijing MDR strains were also observed.

INH or SM resistance was not uncommon, and most RMP-resistant strains were also associated with SM and INH resistance, resulting in MDR. These findings were consistent with a previous report in Ho Chi Minh city in Viet Nam [9]. The high prevalence of primary resistance to INH and SM (28.2% and 28.2%, respectively) and moderate prevalence of RMP resistance and MDR (4.9% and 4.5%, respectively) shown in our study might be considered noteworthy, when comparing with those of South East Asian region (10.3%, 8.9%, 3.4%, and 2.8%) [7], and of China (16.0%, 27.7%, 6.7%, and 5.7%) [18]. In this situation, the use of a regimen with RMP for only 2 months of the intensive phase, which is still accepted in Viet Nam, may pose the risk for poor treatment outcome [19] and accumulation of further drug resistance [20].

The association between younger age and anti-TB drug resistance has been reported previously [9,21]. The results of univariate and multivariate analyses performed in our study indicate that primary drug resistance among the younger population may be confounded by the recent transmission of Beijing strains [9,22]. In the current study, living in an old urban area and infection with clustered strains were associated with INH, but not SM, resistance, suggesting that the transmission of INH-resistant strains is concentrated in areas with a high population density, whereas SM-resistant strains are spreading more diffusely throughout the city. Initially, SM was used for treatment of wound infections during the war in Viet Nam in the early 1950s, which may partly explain the widespread development of SM-resistant nonclustered strains, whereas INH was first circulated in 1960s, and RMP was introduced at around 1975 [23,24]. The Beijing genotype was significantly associated with resistance to any drug, INH, and SM, but it was not associated with either RMP resistance or MDR. A direct role of Beijing strains in drug resistance remains controversial [22,25-27].

The spoligotype and VNTR analyses demonstrated that any-drug resistant strains showed a higher tendency for clustering than fully-sensitive strains; and almost half of the MDR strains were clustered and presumably derived from common infection sources or infection with different sources sharing ancestors [16,28]. Three of the MDR strains (13.6%) belonged to the largest Beijing cluster, accounting for approximately 10% of the study population. Although the Beijing genotype was predominant among clustered MDR strains, three non-Beijing genotype strains were closely related to each other based on their VNTR patterns and showed unclassified spoligo patterns resembling EAI5 or EAI4_VNM, a possibly indigenous MTB subtype mainly observed in Viet Nam. Research into the origin and transmission dynamics of these variant MDR strains, as well as their molecular characteristics, may be important, because it is generally believed that the EAI lineage has conferred significantly less drug resistance compared with other genotypes in Asian countries [29,30].

HIV coinfection was significantly associated with only RMP resistance and MDR in multivariate analysis, although it showed significant associations with all types of drug resistance in univariate analysis. This independent association with RMP resistance and MDR has also been reported in other studies [31,32], including one in the northern area [10], but was not observed in a study of the southern area of Viet Nam [9]. The southern study was conducted between 1998 and 2000, when HIV prevalence was low in Viet Nam [33]. This may explain the lower percentage of HIV, compared with ours (2.8% vs. 9.0%), resulting in a low statistical power (20%) [9]. In Hanoi, approximately 25% of injecting drug users tested were HIV positive [31]. Drug use is a risk factor for nonadherent treatment, and it promotes development of drug resistance [34], thus increasing the chance of resistance transmission among the group. HIV coinfection is also associated with pharmacokinetic alteration of RMP, resulting in a 39% reduction of drug concentration [35]. The decreased bioavailability of RMP may contribute to the development of RMP resistance as well. In addition, HIV-coinfected TB patients receiving antiretroviral treatment often suffer from the adverse effects of RMP when an alternative drug is not available, which may cause poor treatment outcomes [36] and facilitate drug resistance. The negative effect of HIV coinfection on RMP resistance, together with the recent spread of Beijing strains associated with INH resistance, may pose a combined risk for the acquisition and transmission of MDR-TB in a large city like Hanoi.

SM resistance was independently associated with smoking, after adjusted for HIV coinfection. The reason for this association is unknown, although smoking is known to be associated with TB [37]. The proportion of PZA resistance tested using the pyrazinamidase assay was low among the total study population [38]. Nevertheless, the proportion of PZA resistance was significantly higher in the MDR group than that in the non-MDR group, indicating a need for evaluation of the susceptibility of MTB strains to this drug.

The clustering rate in Hanoi (43.4%) was high, presumably because our study was conducted in a capital city with high population density and enrolled only patients with smear-positive pulmonary TB. Others have reported relatively lower clustering rates (28.3% in China [39], 37.7% in Zambia [40], and 16.8% in Uganda [41]). However, these studies were conducted in peripheral areas (Zambia) or enrolled patients with smear-negative pulmonary TB (China, Uganda). In addition, it is known that the resolution of 15 MIRU-VNTR for Beijing strains is suboptimal and may overestimate the clustering rate. Addition of more loci to the standard VNTR loci may increase the resolution in a setting where Beijing-genotype strains prevail [42]. Nevertheless, the data can be analyzed using the standard 15 MIRU-VNTR typing method first, since it has been used internationally for a long time [39-41,43,44].

Our study has some limitations. First, we did not have enough information about direct epidemiologic links among clustered patients. In high TB burden countries, however, a TB outbreak is difficult to identify. In addition, we may not have analyzed all representative isolates in Hanoi city. However, the seven districts participating in this study cover old urban, new urban, and suburban areas in this city, and analysis of a relatively large number of isolates definitely provided information that would be useful in the management of drug-resistant TB. Despite the aforementioned limitations, we investigated a variety of host-, bacteria-, and environment-related factors and developed a multidimensional picture of the status of drug-resistance in the studied area.

In conclusion, the transmission status of drug-resistant TB in a large city with a high proportion of Beijing strains, particularly in HIV-prevalent areas, should be carefully monitored to avoid an increase in the incidence of MDR and generation of extensively drug-resistant TB. Drug susceptibility testing should be considered. On the basis of the results, an optimal treatment regimen, together with intensive monitoring of treatment adherence, is suggested to avoid further increases in drug resistance.

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