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[a]					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.

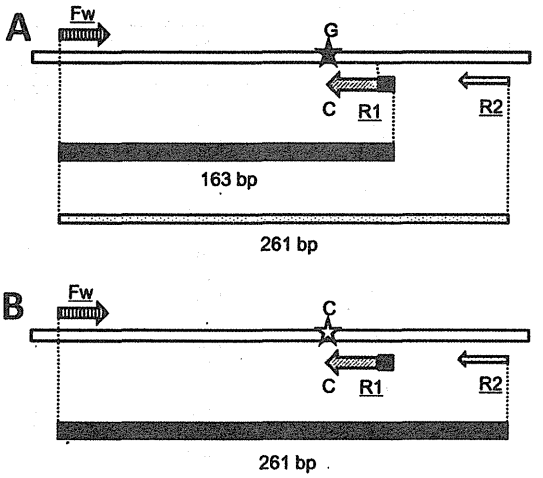


FIG 1 PCR primers and products of *Rv0679c*-targeting multiplex PCR for Beijing lineage discrimination. (A) In the Beijing sample, the 163-bp product is amplified more dominantly than is the 261-bp product. (B) In the non-Beijing sample, 163-bp product is not amplified because of the mismatch of the 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.

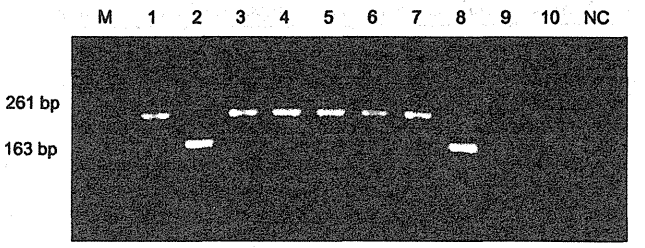


FIG 2 Electrophoresis results of the multiplex PCR products. Lane M, 50-bp ladder DNA size marker; lane 1, *M. bovis* BCG Tokyo 172 (non-Beijing lineage control) strain; lane 2, *M. tuberculosis* OM-9 strain (Beijing lineage control); lane 3, *M. tuberculosis* H37Rv; lane 4, *M. africanum* ATCC 25420; 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.

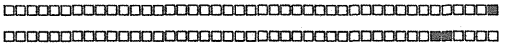
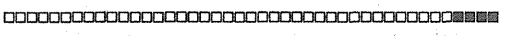
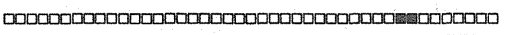
In strain C, the C185T SNP was observed, and in T17, a cytosine was inserted at position 92. In *M. canettii* CIPT 140010059, two SNPs and a codon insertion, ACC at position 154, were observed.

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.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the U.S.-Japan Cooperative Medical Science Programs from the Ministry of Health, Labor, and Welfare of Japan (to Y.S.), by the Global Center of Excellence (COE) Program "Establishment of International Collaboration Centers for Zoonosis Control," Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) (to Y.S.), by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from MEXT (to Y.S.), and by a grant for the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University by MEXT (to Y.S., C.N., and T.M.), as well as by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (JSPS) (to Y.S. and C.N.).

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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.

Citation: Hang NTL, Maeda S, Lien LT, Thuong PH, Hung NV, et al. (2013) Primary Drug-Resistant Tuberculosis in Hanoi, Viet Nam: Present Status and Risk Factors. PLoS ONE 8(8): e71867. doi:10.1371/journal.pone.0071867

Editor: Igor Mokrousov, St. Petersburg Pasteur Institute, Russian Federation

Received May 24, 2013; **Accepted** July 10, 2013; **Published** August 13, 2013

Funding: This work was supported by a grant from the Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT, Japan. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist

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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

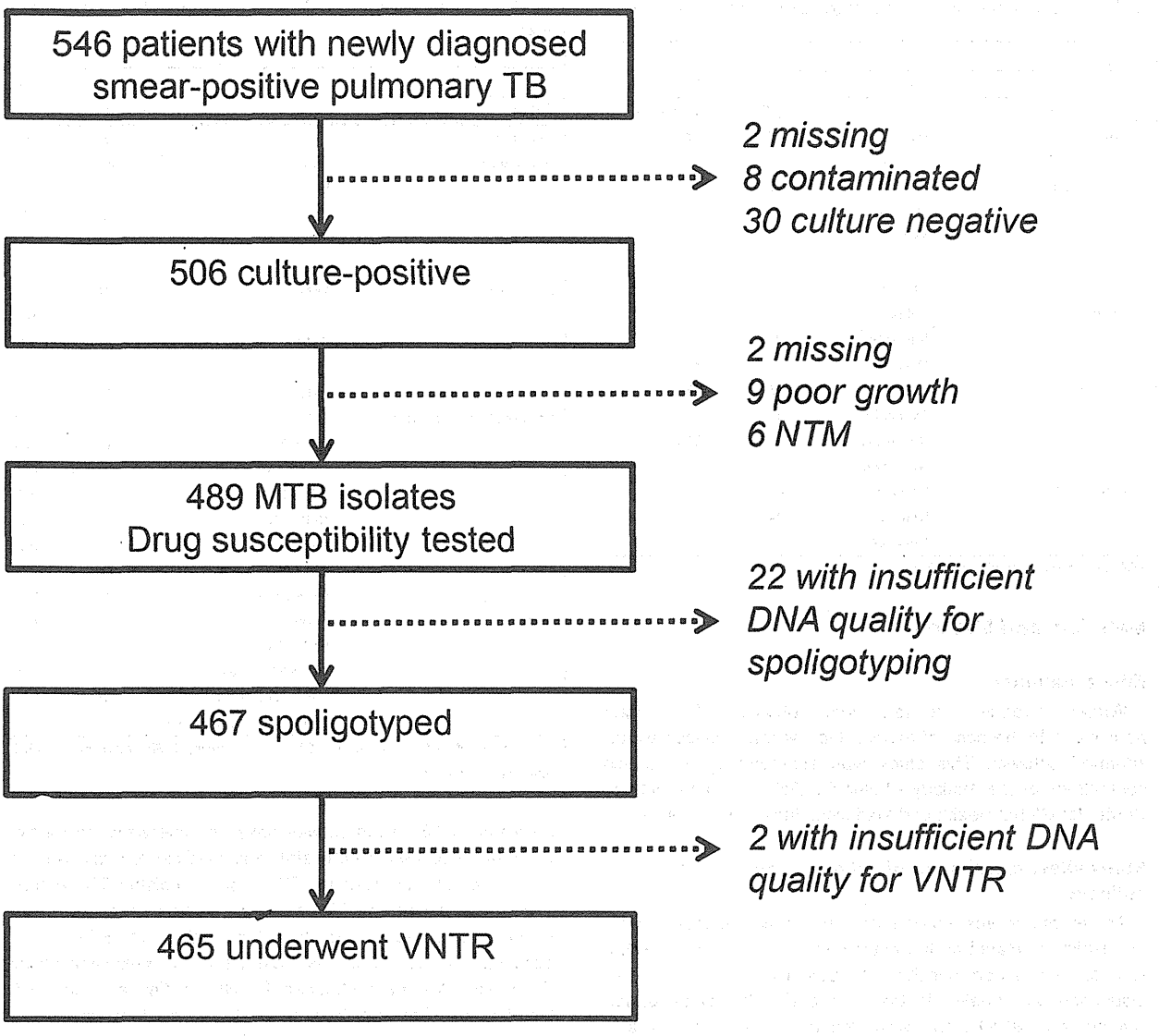

Figure 1. Study flow. TB: tuberculosis; MTB: *Mycobacterium tuberculosis*; NTM: nontuberculous mycobacterium; VNTR: variable numbers of tandem repeats; DNA: deoxyribonucleic acid.
doi: 10.1371/journal.pone.0071867.g001

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.

Acknowledgements

The authors would like to thank Dr. Bui Thi Nguyet, Ms. Vu Thi Xuan Thu, Dr. Pham Thu Anh (Hanoi Lung Hospital), Dr. Phan

Thi Minh Ngoc, Ms. Nguyen Thi Ha (NCGM-BMH Medical Collaboration Center), and all the healthcare staff of relevant district TB centers for supporting site implementation.

Author Contributions

Conceived and designed the experiments: NTLH LTL SM PHT NVH VCC SS HE NK. Performed the experiments: SM TBT NPH KTTN. Analyzed the data: NTLH AN TM NK. Contributed reagents/materials/analysis tools: NTLH LTL SM PHT NVH TBT NPH VCC KTTN SS HE NK. Wrote the manuscript: NTLH SM NK.

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Hung et al. BMC Research Notes 2013, 6:444
http://www.biomedcentral.com/1756-0500/6/444

BMC Research Notes

RESEARCH ARTICLE Open Access

Clonal expansion of *Mycobacterium tuberculosis* isolates and coexisting drug resistance in patients newly diagnosed with pulmonary tuberculosis in Hanoi, Vietnam

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Abstract

Background: Newly diagnosed patients without anti-tuberculosis (TB) treatment histories have not often undergone drug susceptibility testing (DST), but have received the standard treatment regimen without information about their DST profiles in many countries with inadequate resources.

Methods: We collected 346 clinical isolates from previously untreated patients with smear-positive active TB in Hanoi, the capital of Vietnam. Of these, 339 were tested for susceptibility to four first-line anti-TB drugs, including isoniazid (INH), rifampicin (RMP), streptomycin (SM), and ethambutol (EMB), using the proportion method. A pyrazinamidase (PZase) test was used to assess pyrazinamide (PZA) resistance. Results of the culture-based drug susceptibility tests were confirmed by those from reverse hybridization-based line probe assays (LiPAs) that detected mutations associated with RMP, INH, PZA, and fluoroquinolone (FQ) resistance. To investigate a diversity of these strains, IS*6110*-probed restriction fragment length polymorphisms (RFLPs) were analyzed. Nucleotide sequences for *furA-katG* and *fabG1-inhA* operons, transcription units responsible for INH resistance, were also determined.

Results: Of the isolates tested, 127 (37.5%) were resistant to at least one of the four drugs, which included 93 (27.4%) isolates that were resistant to INH. RFLP analysis identified four clusters defined by similarity of the band patterns, which accounted for 46.1% of the tested isolates. Among the clustered isolates, 37.7% were resistant to INH, most of which (85.4%) carried a g944c mutation, which causes an S315T amino acid substitution, in the *katG* gene.

Conclusions: Our results suggest that drug-resistant strains, particularly those with INH resistance characterized by a single mutation, S315T, are spreading in Hanoi, Vietnam. When RMP resistance is combined with this setting, patients are not easily cured by conventional short-term treatment. We will need to carefully monitor these trends and search for the origins and transmission routes of these strains.

Keywords: Primary drug resistance, Isoniazid, Gene mutation, Restriction fragment length polymorphism, Vietnam

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Background

The drug susceptibility profiles of clinically isolated *Mycobacterium tuberculosis* (MTB) strains, particularly those from previously untreated patients, have not been included in clinical practice in many countries with inadequate resources. A single standard anti-tuberculosis (TB) treatment without information regarding drug susceptibility is prone to failure or relapse, as initial drug resistance increases the chance of acquiring additional drug resistance [1].

Molecular fingerprinting of MTB strains has been used extensively and is crucial for elucidating the transmission routes of drug-resistant TB [2,3]. A rapidly developing large city is often accompanied by overcrowding and a floating population, and it is often not easy to identify the epidemiological link between TB cases. Nevertheless, the molecular epidemiological techniques are useful for providing insights into the spread patterns of MTB on site and can thus aid in enhancing TB control activities in the entire city.

Vietnam is a Southeast Asian country stretching over 1,800 km from north to south. It is one of 22 high-burden countries worldwide, and its TB prevalence remains high (323 per 100,000 in 2011) [4]. Vietnam reported an incidence of 2.7% multi-drug resistant-TB among new cases in a 2006 survey (95% confidence interval: 2.0–3.6) [5].

The northern and southern regions of Vietnam have also been under different health policies for more than 20 years. It remains unclear whether entire profiles of MTB isolates obtained in one area are equally useful throughout the country. An earlier report [6] suggested differences in genotypes and drug susceptibility patterns between isolates obtained in distant regions of Vietnam.

Although the status of primary drug resistance has been reported in some areas of Vietnam [7-9], molecular biological approaches to this issue have not yet been completely exploited. Thus, we analyzed the profiles of drug susceptibility testing (DST), drug resistance genes, and fingerprint patterns of MTB isolates obtained from 339 previously untreated patients with smear-positive active TB in Hanoi, the capital of Vietnam.

Methods

Ethics statement

A written informed consent was obtained from each participant. The study was approved by ethical committees of the Ministry of Health, Vietnam and National Center for Global Health and Medicine, Japan.

Clinical isolates from acid-fast bacilli (AFB)-positive sputum

Clinical isolates were consecutively collected from previously untreated patients with AFB-positive active TB in Hanoi city between August 2007 and August 2008. At least two sputum specimens were collected from each patient; one was for a smear test and the other was used for culture in the Department of Microbiology of the Hanoi Lung Hospital. Specimens were decontaminated and homogenized with 0.5% NALC–2% NaOH and subsequently inoculated on Löwenstein–Jensen media. MTB isolates were transferred to the Molecular Biology Laboratory of the National Lung Hospital and subjected to MTB identification using niacin and nitrate tests, DST, and other molecular epidemiological tests.

Drug susceptibility testing (DST)

DST was performed using the proportion method based on World Health Organization (WHO) guidelines [10]. The test medium contained rifampicin (RMP; 40 μg/mL), isoniazid (INH; 0.2 μg/mL and 1.0 μg/mL), ethambutol (EMB; 2.0 μg/mL), and streptomycin (SM; 4.0 μg/mL). Drug resistance was defined as ≥1% colony growth compared with a drug-free control of Löwenstein–Jensen medium.

Pyrazinamidase (PZase) assay

PZase activity was determined by Wayne's method with minor modifications [11,12]. As a positive control, we used the MTB H37Rv strain that is susceptible to pyrazinamide (PZA) and is positive for PZase. As a negative control, we used the *M. bovis* BCG strain that is resistant to PZA and is negative for PZase.

Isolation of genomic DNA

Genomic DNA from MTB was extracted using the original method described [13], with slight modifications [14]. Approximately 400 μl of a bacterial suspension in TE buffer was heated at 80°C for 20 min to kill bacteria. First, 50 μl of lysozyme (10 mg/ml) was added followed by incubation at 37°C for 1 h. Subsequently, 75 μl of SDS/proteinase K was gently mixed followed by incubation at 65°C for 10 min. In addition, 100 μl of 5-M NaCl and 100 μl of CTAB/NaCl solution were thoroughly mixed and incubated for 10 min at 65°C. An equal volume (approximately 750 μl) of chloroform/isoamylalcohol was added, and the mixture was centrifuged for 5 min at 12,000× g. The aqueous supernatant was carefully transferred to another tube. Total DNA was precipitated in isopropanol and was dissolved in 0.1× TE buffer.

Line probe assays (LiPAs)

Reverse hybridization-based LiPAs were used to confirm the results of DST and to detect mutations associated with resistance to RMP [15], INH [16], PZA [12], and fluoroquinolone (FQ) [17]. To detect mutations associated with RMP resistance, 5 oligonucleotide probes were used to hybridize to wild-type sequences and 4 probes to mutation sequences of the *rpoB* gene. For INH resistance,

41 oligonucleotide probes were designed to cover mutations in the regions of *katG* (35 probes), *furA* (2 probes), *fabG1-inhA promoter* (2 probes), and *fabG1* (2 probes). The details and performances of these tests have been reported in the references described above [12,15-17].

Restriction fragment length polymorphism (RFLP)

Experimental procedures for bacterial growth, DNA extraction, DNA digestion with *Pvu*II (Takara Bio Inc. Otsu, Japan), electrophoresis on a 1% agarose gel, and Southern blotting and membrane hybridization with a peroxidase-labeled 245-bp IS*6110* probe were performed using standardized methods [18] with slight modifications [14]. The hybridized probe was visualized with an ECL detection system (Amersham Biosciences). Fingerprinting images were analyzed with Fingerprinting™ II software (Bio-Rad Laboratories, Inc., Hercules, CA), and percent similarity among the isolates was determined according to the supplier's instructions. To classify strains into the same family on the basis of their genotyping profiles, a similarity index of 70%, slightly more stringent than 65% used in a previous report [19] was chosen in this study. Normalization was performed using molecular weight standards and the IS*6110*-fingerprinting patterns of two isolates run on each gel. Isolates with fewer than five IS*6110* copies were excluded from the cluster analysis.

DNA sequencing of INH resistance-related genes

The *furA-katG* operon and its upstream region were amplified by PCR using the specific primers and conditions described previously [16]. The primers used were −129furA (5′-GCTCATCGGAACATACGAAG-3′) and katG +50 (5′-GTGCTGCGGCGGGTTGTGGTTGATCGGCGG-3′). The *fabG1-inhA* operon and the upstream region of the *fabG1-inhA* operon were also amplified using previously reported primers [16]: −200fabG1 (5′-TTCGTA GGGCGTCAATACAC-3′) and inhA +40 (5′-CCGAA CGACAGCAGCAGGAC-3′). PCR products were used as templates for direct DNA sequencing. To detect mutations, DNA sequences were compared with those of H37Rv using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan).

Statistical analysis

Chi-square tests were used to compare proportions between two groups. Kappa statistics were used to determine the agreement between two tests. The following guidelines were used to interpret kappa coefficients: <0, poor agreement; 0–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; and 0.81–1.00, very good. *P* values <0.05 were considered statistically significant, unless otherwise noted. The Bonferroni correction was used when comparing the results for multiple drugs. JMP version 9.0.0 (SAS Institute, Inc., Cary, NC, USA) statistics software was used for analysis.

Results

Clinical isolates from AFB-positive sputum

Clinical isolates were collected from 346 consecutive previously-untreated patients with AFB-positive active TB in Hanoi, Vietnam. Of these patients, 270 (78.0%) were male, and their median age was 38 years (range: 17–84 years). Coinfection with HIV was found in 31 patients (9.0%).

Drug susceptibility profiles of MTB isolates

Sputum samples from 346 smear-positive patients were cultured, from which 339 MTB isolates (98.0%) were obtained. DST information for seven patients was not available. Among these, 127 (37.5%) were resistant to at least one of the four drugs tested; 93 (27.4%), 19 (5.6%), 96 (28.3%), and 11 (3.2%) isolates were resistant to INH, RMP, SM, and EMB, respectively; and 17 (5.0%) were multidrug-resistant (MDR) strains (Table 1). The PZase assay revealed that 8 (2.4%) of the 339 isolates were negative for PZase and were considered to be resistant to PZA (data not shown).

Eighty-eight INH-sensitive and 64 INH-resistant strains were randomly selected. LiPAs for RMP and INH were performed to confirm consistency with the results of culture-based DST and to identify profiles of genetic mutations associated with resistance to these drugs. Agreement between LiPAs and conventional DST was good or very good (kappa = 0.80 for RMP and kappa = 0.84 for INH 0.2 μg/mL; table not shown).

Mutations for RMP included *rpoB*:H526D, H526Y, S526D, and S531L. Only 11 isolates had one of these mutations, and 4 undefined RMP mutations were also observed in our study.

Mutations for INH were mostly *katG*:S315T (data not shown). This was more widely confirmed by subsequent DNA sequencing around the *katG* and *inh* genes (Additional file 1). LiPA for PZA was compared

Table 1 Drug susceptibility profiles of MTB isolates from previously-untreated patients

Patterns		n	% (95% CI)
Sensitive with all drugs		212	62.5 (57.3 - 67.5)
Any resistance	Subtotal	127	37.5 (32.5 - 42.7)
	INH	93	27.4 (23.0 - 32.4)
	RMP	19	5.6 (3.6 - 8.6)
	SM	96	28.3 (23.8 - 33.3)
	EMB	11	3.2 (1.8 - 5.7)
MDR		17	5.0 (3.2 - 7.9)

N = 339 isolates.
INH concentration (0.2 μg/mL).

with the results of the PZase assay; their consistency was moderately high (kappa = 0.55; table not shown). LiPA for FQ identified only 1 mutated strain carrying *gyrA*:A90V.

IS6110-probed RFLP and drug susceptibility

Sufficient DNA was extracted from 317 of the 339 isolates. Their IS*6110*-probed fingerprinting patterns are shown in Figure 1. Four clusters were identified when defined by >70% similarity in their RFLP patterns. These clusters accounted for 146 (46.1%) of the total isolates: 57 (18.0%) in cluster I; 70 (22.1%) in cluster II; 12 (3.8%) in cluster III; and 7 (2.2%) in cluster IV. Each cluster consisted of isolates collected from at least three districts in the city of Hanoi (data not shown).

Within these four clusters, 55 and 49 (37.7% and 33.6%) of the isolates were resistant to INH at 0.2 μg/mL and 1.0 μg/mL, respectively; these proportions were significantly higher than the 19.9% and 17.0% among the non-clustered isolates ($P = 0.0004$ and $P = 0.0006$,

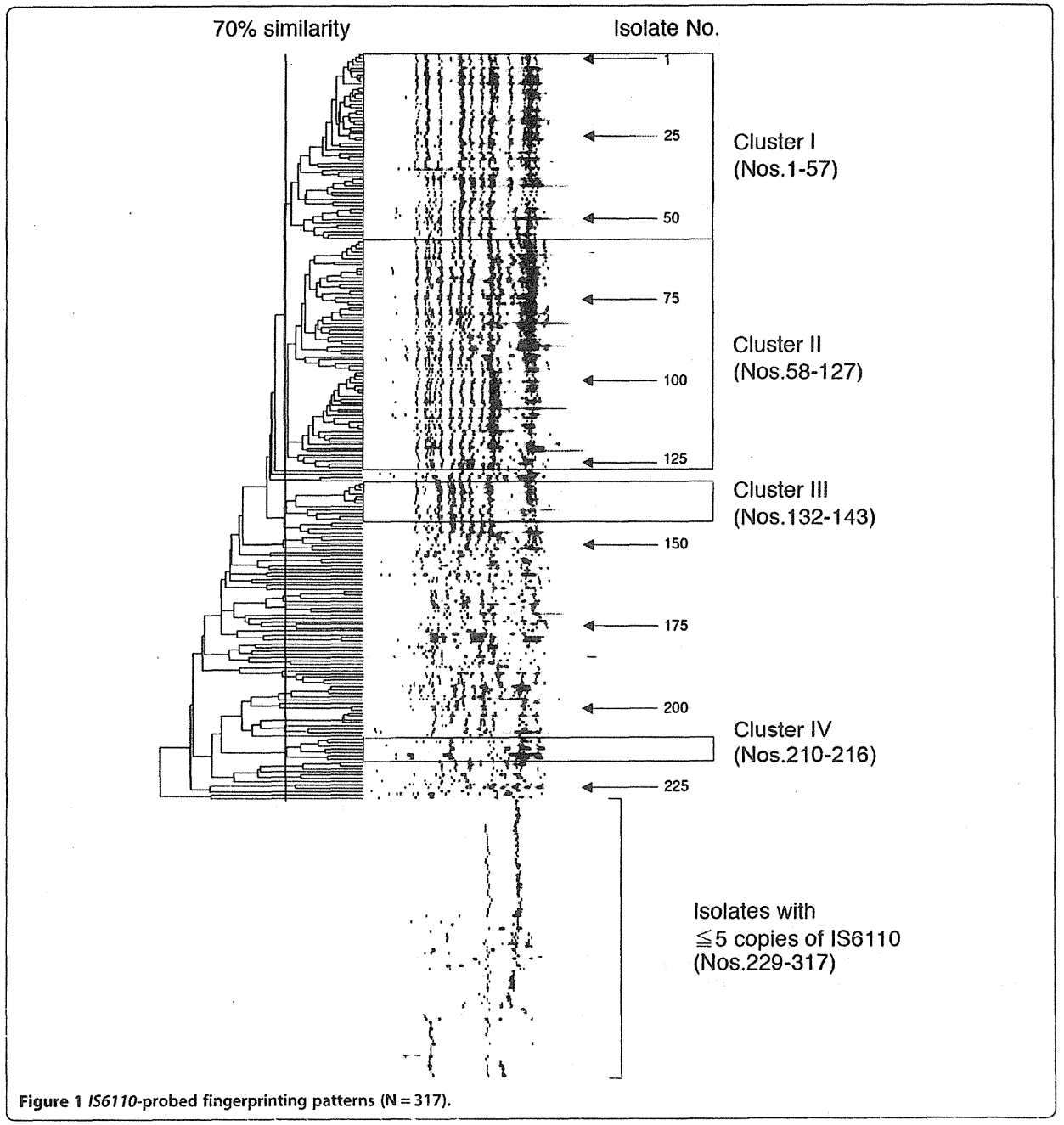

Figure 1 *IS6110*-probed fingerprinting patterns (N = 317).

respectively; Table 2). In fact, in the small clusters III and IV, the majority of the isolates were highly resistant to INH at 1.0 μg/mL (Additional file 1: Table S1). Although the proportions of isolates in these clusters that were resistant to RMP and other drugs also tended to be higher than those of non-clustered isolates, these differences were not significant, based on multiple comparisons statistical testing (Table 2). In addition, there were no significant associations between the clusters and specific *rpoB* mutations (data not shown).

Gene mutations responsible for INH resistance

Among all the INH-resistant isolates noted above, possible mutations within the *furA-katG* operon, the *fabG1-inhA* operon, and their upstream regions were investigated by PCR-based nucleotide sequencing (Additional file 1: Table S1).

Of 89 INH (0.2 μg/mL)-resistant isolates, 76 (85.4%) carried a g944c mutation (AGC to ACC) that caused an S315T amino acid substitution in the *katG* gene and 70 (92.1%) of the isolates that carried the g944c mutation were highly resistant to INH at 1.0 μg/mL. Furthermore, g204c mutations in the *furA* operon were detected in 13.6% of all the isolates and were frequently accompanied by the g944c mutation in the *katG* gene, although this variant itself was not directly associated with culture-based INH resistance ($P = 0.072$; table not shown). In the upstream region of the *fabG1-inhA* operon, c-15t was observed in 7 isolates, and minor variations with <1% were observed within the *fabG1-inhA* operon. The combination of a g944c mutation in the *katG* gene with c-15t in the *inhA* promoter was observed only in 1 isolate.

Discussion

We investigated the drug susceptibility profiles of clinical isolates obtained from previously untreated patients with active pulmonary TB in Hanoi, the northern largest city in Vietnam, and found that a quarter of these isolates were highly resistant to INH, most of which had a single S315T mutation in the *katG* gene. These isolates with primary resistance to INH were enriched in the clusters identified by RFLP. They probably originated from a few genetically related clones and were recently transmitted into and spread within this area of Vietnam.

Table 2 The relationship between drug resistance and clustering of the clinical isolates in Hanoi

Resistant to	MTB isolates		P value*
	Clustered	Non-clustered	
	N = 146	N = 171	
INH (0.2 μg/mL)	55 (37.7%)	34 (19.9%)	0.0004
INH (1.0 μg/mL)	49 (33.6%)	29 (17.0%)	0.0006
RMP	13 (8.9%)	6 (3.5%)	0.0437
SM	49 (33.6%)	41 (24.0%)	0.0592
EMB	7 (4.8%)	4 (2.3%)	0.2338

*P values by chi-square test. P < 0.01 was regarded as statistically significant after considering multiple comparisons.

Among the isolates with resistance to the first-line drugs tested in this study, a high proportion of primary INH resistance (27.4%) was rather characteristic. This resistance level was higher than the average (19.1%) obtained during a 2006 nation-wide survey [5]. Such a high level of primary resistance is a serious concern because INH is a key drug by which newly diagnosed TB patients can be successfully treated. In Vietnam, culture-based DST has not yet been routinely performed for previously untreated patients, and the standard regimen for these patients remained 2S(E)HRZ/6HE for a long time [20]. In the years when RMP-based treatment was not easily accomplished during the maintenance phase in areas with inadequate resources, this regimen had certain significance and was thus endorsed by the WHO until recently [21].

However, when DST results are unknown and the above standard regimen is used for INH-resistant TB, treatment during the maintenance phase is no more than EMB monotherapy, which could increase the chances of failure, early relapse, and additional drug resistance [21,22]. Prescribing 2RHEZ/4RHE, a regimen that includes 6 months of RMP, has also been recently approved by the national TB program in Vietnam. Because treatment outcomes are largely affected by locale-specific factors, including patient adherence to the regimen and drug resistance profiles of the prevailing strains, further studies will be needed to confirm the optimal regimens in Vietnam [23].

To reduce the likelihood of failure, relapse, and additional acquired drug resistance in major cities, updating clinical laboratories for DST is an urgent need [21]. In addition to DST for first-line drugs, detecting resistance to second-line drugs, including FQ, has also become important recently [24], although the proportion appeared to be low (<1%) in our study. Even in a resource-poor setting, as per timely DST results, health care staff should treat and intensively follow up those patients with drug-resistant TB with the aim of complete cure in most of these cases and to prevent further spread of MDR-TB and generation of extensively drug-resistant TB.

Genetic analysis of our MTB isolates demonstrated that >85% of the INH resistance (92% with high-level resistance) was caused by a S315T mutation in the *katG* gene. The predominance of this mutation in INH resistance has been observed in most of the areas with high TB prevalence, although the proportion (85%) in our study was relatively high compared with what was reported in other studies [25-27]. Continuous use of INH may cause additional mutations and induce higher levels

of resistance [27,28]. Rapid detection of INH resistance at an early phase is important to break this chain of acquiring additional resistance. Predominance of the S315T mutation is potentially advantageous for providing molecular DST in a resource-limited setting because it might entice manufacturers to develop a simplified, maintenance-free genetic test specialized for detecting the relevant mutations at a reasonably low cost [29].

RFLP analysis demonstrated that primary resistance to INH was more often observed in clustered isolates than in non-clustered isolates. Resistance to other drugs also appeared to be associated with these clustered isolates, although the tendencies were not as clear as that for INH. This indicates that expansion of INH-resistant isolates presumably originated from a few genetically related clones and that they were transmitted into the city of Hanoi and they spread widely within a relatively short period. Rapid expansion of genetically related strains may also explain why a single INH-resistant mutation, S315T, was predominantly detected in this area.

In our study, other genotype data for these clinical isolates were not available. However, in Vietnam, particularly in the southern region, two families of strains, designated the Beijing genotype and a presumably indigenous East-African Indian (EAI) genotype, are known to be predominant [30]. According to the literature, strains with ≥15 IS*6110* copies may indicate typical Beijing strains [31], whereas most of the EAI strains in Vietnam have <5 copies [30]. The copy numbers and RFLP pattern profiles in large cluster II in our study were definitely consistent with those of Beijing strains, whereas approximately one-fourth of the isolates had a few copies that are observed in EAI. IS*6110* copy numbers in other clusters were those between these two families. An earlier study demonstrated that copy numbers of northern strains in Vietnam were relatively smaller than those of southern strains in which typical Beijing genotypes are frequently observed [6]. We will need to further characterize the strains originating from the Hanoi area in future genotypic studies.

Our study had some limitations. The isolates analyzed in this study were collected from sputum smear-positive patients who visited TB clinics. Therefore, we may have only extracted features of MTB isolates from moderate to severe pulmonary TB cases. Nevertheless, understanding the current status of highly transmissible smear-positive TB is a priority for TB control because Vietnam is one of the high TB burden countries.

Conclusions

High levels of primary resistance to INH and emerging RMP resistance may be closely related to the problems of a rapidly developing city, such as the distribution of young workers with low incomes, undernutrition, poor hygiene, and crowding in a densely populated urban area with a floating population. Private acquisition and inappropriate use of anti-TB drugs through unofficial distribution routes are also difficult to manage in a large city such as Hanoi. It will be necessary to curb the transmission of drug-resistant MTB by considering effective counter measures. We will need to carefully monitor these trends further and search for the origins and transmission routes of these Southeast Asian MTB strains.

Additional file

Additional file 1: Table S1. Drug susceptibility testing and DNA sequencing of *M. tuberculosis* clinical isolates (N = 317).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NVH supervised on-site implementation of this study and drafted and revised the manuscript. HA performed the experiments and participated in technical transfer and supervision. TTBT carried out the drug susceptibility tests. TK performed the experiments. NTLH supervised on-site implementation of this study and drafted and revised the manuscript. SS and PHT monitored on-site data collection. LTL conceived and supervised this study. NK conceived the study, analyzed and interpreted data, and drafted and revised the manuscript. All authors read and approved this manuscript.

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Acknowledgments

This work was supported by a grant from the Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT, Japan. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Dr. Bui Thi Nguyet, Ms. Vu Thi Xuan Thu, Dr. Pham Thu Anh (Hanoi Lung Hospital), Dr. Phan Thi Minh Ngoc, Ms. Nguyen Thi Ha (NCGM-BMH Medical Collaboration Center), and all healthcare staff of the relevant district TB centers for supporting site implementation.

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Received: 3 May 2013 Accepted: 29 October 2013
Published: 5 November 2013

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