showed that the incidence of TB infections examined by IGRA (in most of the cases IGRA ELISA was used) was very low in NTM patients in MTB non-endemic countries (Van Leeuwen et al. 2007; Adams et al. 2008). In contrast, significant percentages of NTM were positive by IGRA in Taiwan and Korea (Wang et al. 2007; Ra et al. 2011). Given the variety of clinical courses of NTM, the effect of LTBI on the clinical and/or laboratory features of NTM should be evaluated.

It is well known that CD4 cells and Th1-mediated signaling molecules and their pathways are important in the defense against NTM infection (Griffith et al. 2007). Recently, it was found that osteopontin (OPN), a pro-inflammatory cytokine secreted by a wide variety of cells including T cells and macrophages and elevated in tuberculosis, is linked with CD4 T helper (Th1) cell lineage stimulation and was reported to regulate *Mycobacterium avium* in cattle (Karcher et al. 2008). It was also claimed that OPN expression correlates with effective immune and inflammatory responses in MAC-infected individuals (Nau et al. 2000). We also measured pentraxin-3 (PTX-3), which is associated with the acute-phase response and involved in innate immunity. PTX-3 is produced by mononuclear phagocytes, dendritic cells, and endothelial cells in response to inflammatory signals. PTX3 binds with high affinity to the complement and activates the classical pathway of complement and facilitates pathogen recognition by macrophages. (Garlanda et al. 2005; Inforzato et al. 2012). The plasma PTX-3 level, which was reported to reflect the degree of inflammation in MTB infection (Azzuri et al. 2005), can be highly expressed *in vitro* on human PBMCs and monocytes stimulated with lipoarabinomannan (LAM) (Vouret-Craviari et al. 1997).

The current study was designed to compare various serological markers that are associated with TB, including OPN, PTX-3, leptin and soluble IL-2 receptor (sIL-2R), in NTM patients with or without LTBI to characterize their status.

Materials and Methods

Study population

All the patients that participated in this study were receiving care at Tohoku University Hospital between January 2008 and July 2010. We enrolled 9 patients (N1-9) who met the 2007 American Thoracic Society (ATS) microbiological criteria for pulmonary NTM diseases (Griffith et al. 2007). Six patients diagnosed as active TB (T1-6) were served as disease controls (Table 1). Among them, 2 patients (N7, T3) were withdrawn themselves from the study. NTM and MTB were confirmed from the site of infection by culture. In addition to culture, we repeatedly tested the samples from patients by PCR (Roche amplicon), and MTB was never detected in the NTM group. All the patients were assessed for clinical features, medical history including prior tuberculosis disease, treatment history and chest CT scan finding. Individuals with HIV/AIDS infection or who were receiving immunosuppressive therapy were excluded from the study. Five healthy volunteers who were without any symptoms relevant to active tuberculosis were enrolled as negative controls. The study was approved by the Ethics Committee of Tohoku University Hospital (2007-136; 2007-257). We obtained written informed consent from all the participants. All work was conducted in accordance with the Helsinki declaration. Plasma was obtained from EDTA-containing blood by centrifugation and was aliquoted to cryotubes and stored at −80°C for future use. Simultaneously, PBMCs were isolated over Ficoll-Paque Plus gradient and suspended in RPMI 1,640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml), gentamycin (5 μg/ml) and 10% heat-inactivated FCS (Sigma) for further assay.

All the laboratory data including blood cell counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and sIL-2R were measured at Tohoku University Hospital.

Table 1. Patients profile and result of IFN-γ release assay (IGRA).

Patients ID	Gender	Age (yr)	Diagnosis	Mycobacteria	Duration of illness	IGRA
N1	M	79	NTM	*M. avium*	5 yrs	negative
N2	F	76	NTM	*M. avium*	5 yrs	positive
N3	F	62	NTM	*M. avium*, *M. intracellulare*, *M. fortuitum*	6 months	negative
N4	F	83	NTM	*M. avium*	7 yrs	negative
N5	F	68	NTM	*M. avium*	7 yrs	positive
N6	M	43	NTM	*M. abscesus*	6 months	positive
N8	M	61	NTM	*M. avium*	13 yrs	negative
N9	F	76	NTM	*M. intracellulare*	3 yrs	positive
T1	F	78	ETB	*M. tuberculosis*	2 months	positive
T2	F	71	ETB	*M. tuberculosis*	1 month	positive
T4	M	65	ETB	*M. tuberculosis*	1 month	positive
T5	F	32	ETB	*M. tuberculosis*	1 month	positive
T6	F	57	PTB	*M. tuberculosis*	1 month	positive

NTM, non-tuberculosis mycobacterium; ETB, extra-pulmonary tuberculosis, PTB, pulmonary tuberculosis.

RD1 PCR analysis

Mycobacterial isolates from 6 NTM patients were kindly provided by the central research laboratory of TUH. DNA was extracted from the clinical isolates as described previously (Nakajima et al. 2010). Briefly, glass beads (0.1 mm), the mycobacteria sample (500 μl in TE buffer) and 500 μl Chloroform were put in a 2 ml microcentrifuge tube for and oscillated by a minibeadbeater, at 4,800 rpm for 2 min. The aqueous phase was collected immediately upon centrifugation. DNA was isolated by 80% ethanol precipitation, dissolved in 50-μl sterile distilled water and stored in −20°C until assay. DNA samples were amplified for the RD1 region (150 bp) by PCR analysis using the same primers described previously (Parsons et al. 2002). DNA from the H37RV strain of *M. tuberculosis* was used as a positive control. PCR was performed with 5 μl DNA samples in a total volume of 50 μl of PCR mix, 5 μl 10 × buffer, 4 μl of 2.5 nM dNTP, 1 μl of Taq DNA polymerase and 0.2 μl of each primer (50 pmol/μl). The mixture was denatured at 95°C for 5 min and cycled for 45 times at 94°C for 30 s than 62°C for 45 s and 68°C for 45 s followed by a final 10-min extension at 68°C. The PCR product was visualized by UV transilumination of ethidine bromide staining after separation by 2% gel electrophoresis.

IGRA ELISPOT assay

The assay was performed as described previously (Guio et al. 2010). Freshly isolated peripheral blood mononuclear cells (2.5×10^5 per well) were cultured on plates precoated with antibody against IFN-γ (IGRA ELISPOT. Oxford Immunotech, Oxford UK). After 18-20 hours stimulation of the cells with Early Secreted Antigenic Target-6 (ESAT-6) and CFP-10, the spots were developed according to the manufacturer's instructions. Spot-forming units (SFUs) were counted with an automated ELISPOT reader (KS ELISPOT Carl Zeiss MicroImaging-Germany). The responses were scored as positive if the test wells contained a mean of at least 5 SFUs more than the mean of the negative control wells and was at least twice the mean of the negative control wells.

Inflammatory markers

Anti-TBGL antibody which recognizes glycolipid (maily trehalose 6,6'-dimycolate) of MTB and NTMs was meausered as described (Mizusawa et al. 2008). The plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) as described (Chagan-Yasutan et al. 2009), and the plasma PTX-3 levels were measured in special reference laboratory (SRL, Hachiouji, Japan) (Peri et al. 2000). Plasma levels of leptin were measured as described (Siddiqi et al. 2012).

Statistical analysis

Data were analyzed by statcel2 software (OMS, Tokyo, Japan). Continuous data were compared between groups by Mann Whitney *U* test and significance was considered a p value < 0.05.

Results

Subjects

Profiles of the patients are listed in Table 1. The NTM patients (43 to 83 years old (y.o.)) were infected predominantly by *M. avium complex* (Table 1) and mostly had pulmonary involvement except N6, who had extra-pulmonary involvement (nasal granulomatosis). The duration of illness was variable, ranging from 2 to 148 months. None of them were infected with *M. marinum, M. szulgai* or *M. kansasi,* which are known to possess the RD1 region. All the TB patients included in this study (32 to 78 y.o.) had extra-pulmonary disease except T6 (pulmonary TB). All 5 healthy volunteers (25 to 60 y.o.) were apparently healthy without any TB-related symptoms.

Verification of absence of RD1 region and M. tuberculosis strains

Absence of the RD1 region in the DNA samples from the clinical strains of the 6 NTM patients (N1-N6) was confirmed by PCR analysis. No positive band was observed at the 150 bp position for the RD1 region in any of the clinical isolates (Fig. 1).

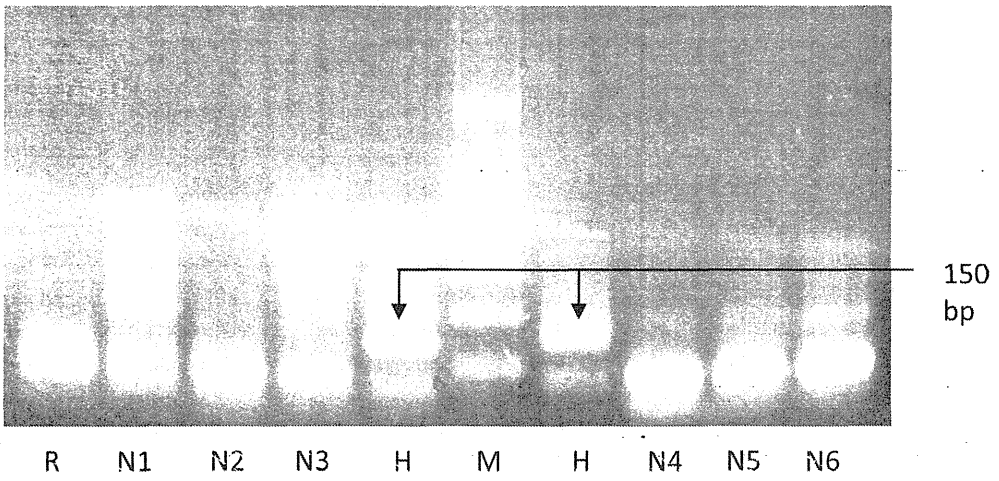

Fig. 1. PCR amplification of RD1 region using DNA obtained from mycobacteria isolated from NTM patients. Arrows indicate the positive bands at 150 bp. N1 to N6, (DNA from 6 NTM patients); H, DNA from *Mycobacterium tuberculosis* (H37RV); M, Marker; R, reagent only.

IGRA and association with the clinical condition

All healthy controls except one were negative for IGRA. Four of 8 NTM patients and all TB patients were positive by IGRA (Table 1). Repeated examination by culture and PCR showed negative for MTB infection in all NTM patients. We further stratified these NTM patients into two subgroups, IGRA-positive as NTM with LTBI (NTM+) and IGRA-negative as NTM without LTBI (NTM−).

Patients in the NTM+ group had pulmonary pathology related to NTM disease based on clinical and radiographic features: consolidation and pulmonary infiltration in N2, both cavity and nodule in N5 and N9 had a combination of bronchiectasis, cavity, consolidation and nodules. Patients of the NTM− group also had typical radiographic features related to NTM infection. The patients of the NTM+ group had symptoms for longer periods (36 to 84 months), whereas the NTM patients had symptoms for variable durations (2 to 148 months), although the difference was not significant. No differences in age or clinical presentation were observed between the NTM+ and NTM− groups. Only patient N5 from the NTM+ group had a history of prior TB infection at the age of 18 that was cured by anti-TB therapy. Among all the NTM patients, only 2 NTM+ patients (N5, N9) had been receiving treatment for NTM infection because of clinical and radiological severities. Patient N5 was treated with rifampicine (RFP), clarithromycine (CAM), ciprofloxacin (CPFX) and ehtambutol (EB) for 6 months starting from July 2001. From May 2004, he was treated again with RFP and CAM, and treatment was continued until October 2005. Since then, he has been treated with different quinolone derivatives including gatifloxacin, CPFX, levofloxacin until June 2006. Finally, the patient was on combined therapy of RFP, CAM and a quinolone derivative until the time of the assay. Patient N9 was also treated with RFP, EB and CAM for 2 months before enrolling in the study. All the other patients hadn't received any drugs for NTM infection. Patients, N5 and N9 had died before the writing of this paper, though detailed information was not available.

Laboratory markers

Laboratory findings are listed in Table 2. There were no significant differences in the conventional markers between the NTM− and + groups. The levels of leptin were apparently lower in the NTM+ and TB groups than in the NTM− group, but no significant differences were found among the groups (Table 2, Fig. 2F).

Inflammatory markers

The data of inflammatory markers are shown (Fig. 2). The TBGL antibody levels are elevated in the NTM+ group and such elevations were unexpectedly not seen in the MTB group, probably because the most of the TB patients were the mild, extra-pulmonary type (Fig. 2A). Patients of the NTM+ group had increased levels of OPN (859 to 1,499 ng/ml) (normal value: < 820 ng/ml; according to Chagan-Yasutan et al. 2009) and the levels were significantly higher

Table 2. Clinical characteristics of patients enrolled in the study.

Laboratory data	Ref. range	Median		
		NTM−	NTM+	TB
RBC (10^6/ul)	3.93-5.03	3.915	3.74	3.76
WBC (/ul)	3.2-9.6	5,250	5,650	4,100
Neutrophil %	31-73	62	58.5	69
Eosinophil %	0-7	1	2	1
Basophil %	0-3	0.5	0.5	0.005
Lymphocyte %	18-51	27	30.5	5
Monocyte %	1-12	8	7.5	5
Platelets (10^3/ul)	155-347	232.5	229.5	277
Hb (g/dl)	11.7-14.8	12.9	11.2	11.7
CRP (mg/dl)	0-0.2	1.2	2.45	0.5
Albumin (g/dl)	4.2-5.3	4.1	3.6	3.7
IgG (mg/dl)	748-1,694	1,320	1,542	1,564
IgM (mg/dl)	33-254	86.5	103	115.5
IgA (mg/dl)	91-391	281	336	295
ESR (30 min) (mm)	(−)	8	11	23
ESR (1 hr) (mm)	(−)	22.5	25	53
KL-6 (U/ml)	105-435	310	315.5	347.5
Leptin (pg/ml)	4,700-32,500	8,105	3,437	4,013

NTM−, IGRA negative non-tuberculosis mycobacterium patients; NTM+, IGRA positive non-tuberculosis mycobacterium patients; TB, tuberculosis patients.

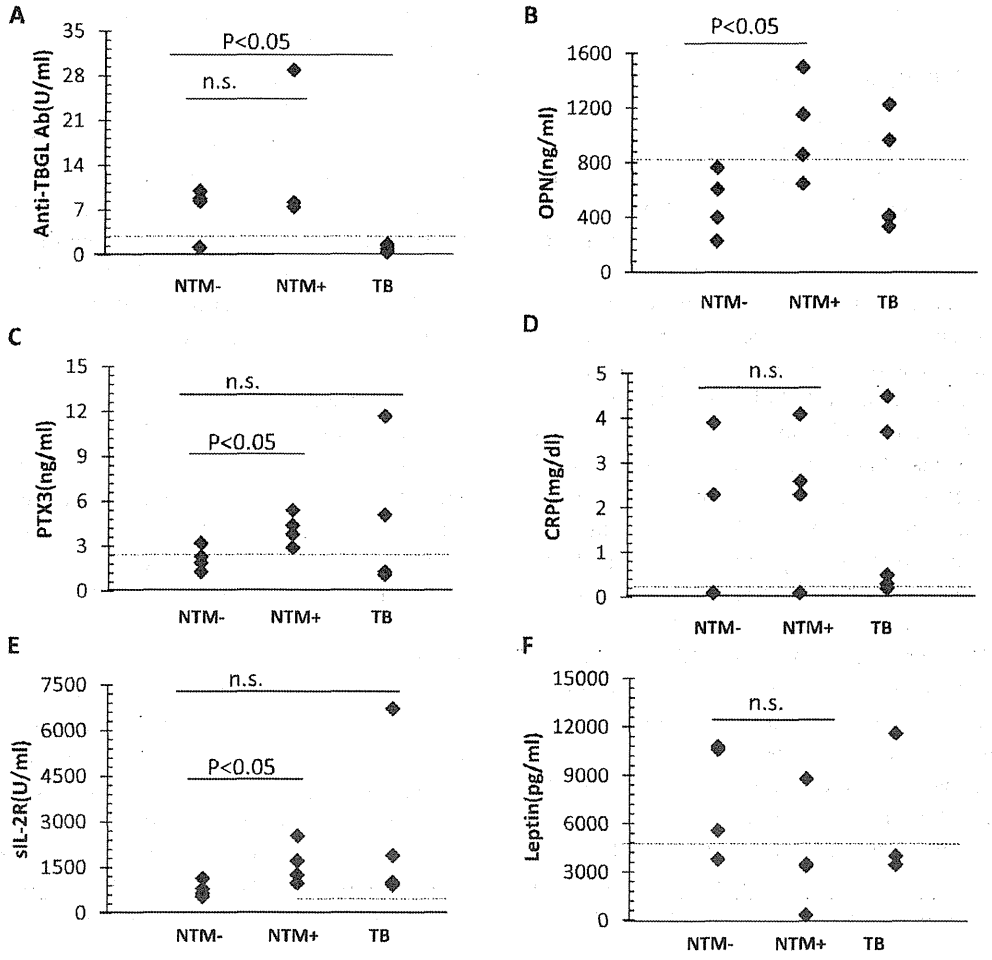

Fig. 2. Comparison of plasma inflammatory molecules.
NTM−, NTM patients without LTBI ($n = 4$); NTM+, NTM patients with LTBI ($n = 4$); TB, active TB patients ($n = 5$).

than those of the NTM− ($p < 0.05$) (Fig. 2B). The OPN titers in patients N5 and N9 were very high 1,150 ng/ml and 1,499 ng/ml, respectively (Fig. 2B). The CRP levels were not different between the NTM+ and NTM− groups, but the PTX-3 levels (normal value: < 2.3 U/ml) and sIL-2R (normal value: 122-496 U/ml) were elevated in all NTM+ patients and the difference from the NTM− group was significant ($P < 0.05$) (Fig. 2C, D and E).

Discussion

In the current study, 4 of 8 NTM patients (50%) were found to be LTBI, and their average age was 74.5 y.o. In addition, PCR analysis showed that all of our NTM isolates lacked the RD1 region. An age-dependent increase of LTBI has already been described in Japan, where 9.8% for those aged 60-69 were IGRA ELISA positive (Mori et al. 2007). The excellence of IGRA ELISA to differentiate NTM and TB infection in children in non endemic countries was reported (Detjen et al. 2007). In Japan, only 1-8% of IGRA ELISA positive rate in Japanese patients with MAC disease was reported (Kobashi et al. 2006; Kobashi et al. 2009). However 34-49% IGRA ELISA positive cases were reported in NTM in endemic countries (Ra et al. 2011). The reasons of high rates of IGRA-positive rates in this study could be explained by IGRA ELISPOT assay employed here. It is known that IGRA ELISA tends to show false negative results among thin elderly people, presumably due to decreased immune levels, whereas IGRA ELISPOT assay might have detected LTBI more sensitively in the elderly patients with NTM disease. It was reported that the long-lasting positive IFN-γ response to antigenic challenge continues for 5 to 10 years following anti-TB therapy (Adams et al. 2008).

NTM+ patients had significantly higher titers of inflammatory markers such as OPN, PTX-3 and sIL-2R, though CRP did not show significant differences (Fig. 2). We also observed sustained high levels of OPN after treatment in NTM+ patients. It is possible that anti-NTM therapy was ineffective because two patients died. Alternatively, persistent elevations of OPN after chemotherapy were already reported by us in AIDS patients treated by anti retroviral therapy (Chagan-Yasutan et al. 2009). Additionally our recent study supports the idea of immune-modulator effect of quinolone which enhance the

production of OPN in human lung epithelial cell line A549 *in vitro* (Shiratori et al. 2012). We assume that the quinolone treatment may be one of the factors of persistent OPN elevation. The increased plasma OPN in TB patients contributed to the disease pathology by activating the IL-12 mediated Th1 immunity (Koguchi et al. 2003). It was also found that OPN expression correlates with an effective inflammatory response and contributes to human resistance against MTB (Nau et al. 2000). In cattle, it was proposed that OPN is a key regulator against *M. avium* (Karcher et al. 2008). Immune responses by *M. avium complex* preferentially depend on the phase of infection in human. Early acute infection causes increased IFN-γ secretion, while the chronic phase has been reported to be associated with copious IL-10 production (Azouaou et al. 1997) with an inclination toward Th2 cytokines (Vouret-Craviari et al. 1997) that may provide protection against chronic diseases. We have already reported that the plasma levels of IFN-γ, OPN and leptins did not show any significant changes between LTBI and non-LTBI health care workers (HCW). Though only LTBI HCWs showed the association of TBGL-IgA antibody titer and serum IFN-γ (Siddiqi et al. in press). Our finding may imply that NTM co-infection with LTBI can synergistically induce large amounts of OPN. The synergistic effect could be explained by the natural resistance associated macrophage protein 1 (NRAMP1) because it was reported as host genetic factor for development of both tuberculosis and NTM, however the involvement of NRAMP1 in OPN production was not studied (Li et al. 2011; Sapkota et al. 2012).

It is also interesting that the PTX-3 levels were significantly higher in the NTM+ group while the CRP levels did not differ. It was documented that 5 of 220 TB contacts who developed active TB within 5 to 12 months of follow-up had elevated levels of PTX-3 (Azzuri et al. 2005). The PTX3 haplotype frequencies significantly differed in TB cases compared to controls, and a protective effect against MTB was found in association with a specific haplotype (Olesen et al. 2007). Hence, Th1 mediated PTX-3 production in mycobacterial infection also warrants further investigation.

In conclusion, frequent LTBI was detected in aged NTM patients, and these patients expressed higher levels of inflammatory markers than NTM without LTBI patients. The low number of patients is the main limitation of this study, but careful observation and extensive therapeutic intervention appear to be necessary in NTM with LTBI patients.

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Conflict of Interest

The authors declare no conflict of interest.

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Genotypes and Characteristics of Clustering and Drug Susceptibility of *Mycobacterium tuberculosis* Isolates Collected in Heilongjiang Province, China[▽]

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For the last decade China has occupied second place, after India, among the top five countries with high burdens of tuberculosis (TB). Heilongjiang Province is located in northeastern China. The prevalence of drug-resistant TB in Heilongjiang Province is higher than the average level in China. To determine the transmission characteristics of *Mycobacterium tuberculosis* strains isolated in this area and their genetic relationships, especially among the Beijing family strains, we investigated their genotypes. From May 2007 to October 2008, 200 *M. tuberculosis* isolates from patients presenting pulmonary TB were analyzed by molecular typing using PCR-based methods: spacer-oligonucleotide typing (spoligotyping), Beijing family-specific PCR (detection of the deletion of region of difference 105 [RD105]), and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) analysis. Different combinations of MIRU-VNTR loci were evaluated to define the genotypes and clustering characteristics of the local strains. We found that Beijing family strains represented 89.5% of the isolates studied. However, the rates of multidrug-resistant (MDR) *M. tuberculosis* among Beijing and non-Beijing family strains were not statistically different. The 15-locus set is considered the optimal MIRU-VNTR locus combination for analyzing the *M. tuberculosis* strains epidemic in this area, while the 10-locus set is an ideal set for first-line molecular typing. We found that the clustering rate of all the *M. tuberculosis* isolates analyzed was 10.0% using the 15-locus set typing. We conclude that the Beijing family genotype is predominant and that highly epidemic TB and MDR TB are less likely associated with the active transmission of *M. tuberculosis* in the study area.

Tuberculosis (TB) remains a major public health threat worldwide. China has occupied second place, after India, among the top five high-burden countries for the last decade (http://www.who.int/tb/en). Although both the incidence and prevalence of TB in China have shown a steady decline in recent years, it remains a leading notifiable infectious disease (http://www.moh.gov.cn). Currently, the spread of drug-resistant TB, especially multidrug-resistant (MDR) TB, in China, presents a major challenge. The most up-to-date data from the World Health Organization (WHO) indicates that the rate of MDR TB in China was 8.3% (the rates of primary and acquired MDR TB were 5.7% and 25.6%, respectively) in 2007 (50), significantly higher than the global average rate (3.6%). The higher levels of TB prevalence and drug resistance have become the main public health concern of the Chinese government.

Heilongjiang Province, located in northeastern China, is one of the regions where the prevalence of both TB and drug-resistant TB is higher than the average level in China. The most recently updated epidemiological data, from 2007 to 2008, show that the rates of primary and acquired MDR TB were 18.3% and 37.8%, respectively, in Heilongjiang Province (our unpublished data). The reasons for the high prevalence and drug resistance of TB in Heilongjiang Province are still unknown and should be investigated to facilitate control of the TB epidemic in this area and throughout China.

In several Asian countries with high TB rates, a unique genotype of *Mycobacterium tuberculosis*, known as the Beijing family genotype, has been found to be the dominant genotype (3, 17, 34). During the last decade, Beijing family strains have been spreading in various geographic locations worldwide and now account for more than a quarter of all TB cases worldwide (12). Possible associations of the epidemic caused by this genotype with its drug resistance (1, 41) and its high adaptability to the host intracellular environment (8) have been reported. In China, Beijing family strains have spread widely; however, the proportion of Beijing family strains in Heilongjiang Province remains unknown. It is thus unclear if the high prevalence and high drug resistance of epidemic TB are directly related to the spread of Beijing family strains. The answers to these

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TABLE 1. The profile of spoligotypes and RD105 deletion of the *M. tuberculosis* isolates (n = 44)

SIT no.[a]	Spoligotype[b]	Label	No. of isolates[c]	RD105 deletion
1		Beijing	39	+
190		Beijing	1	+
1793		Undesignated	1	—
—		New	1 (2460)	+
—		New	1 (2488)	—
—		New	1 (2495)	—

[a] —, unassigned in the SpolDB4 database.
[b] The black and white boxes indicate the presence and absence of the specific spacer, respectively.
[c] The identification number of the isolate is given in parentheses.

questions would be very helpful in achieving effective control of TB in Heilongjiang Province as well as throughout China.

To determine the spreading characteristics and the genetic relationships among the *M. tuberculosis* isolates in a given geographic region, a suitable genotyping method is the most important means, i.e., one that is discriminatory, economical, and reproducible. Spacer-oligonucleotide typing (spoligotyping) is a PCR-based method which is simple, highly reproducible, and applicable to a digital format (22). Spoligotyping possesses the most features of an ideal typing method and is the gold standard for identifying strains as belonging to the Beijing family. However, due to the genetic homogeneity of this family, this method is uninformative in the areas where the Beijing family is prevalent (47) and is therefore limited to use as a first-line typing method.

A genotyping method based on mycobacterial interspersed repetitive unit-variable-number tandem repeats (MIRU-VNTR), which is technically flexible to facilitate interlaboratory comparison and database management, is well accepted and has recently been adapted to high-throughput techniques (7, 39). The digital format of MIRU-VNTR patterns makes this method suitable for the global study of the molecular epidemiology of *M. tuberculosis*. A number of studies have investigated the usefulness of different VNTR loci for typing *M. tuberculosis* and have shown that the discriminatory power of this method may be comparable to that of IS*6110* typing, which is the most reliable method for the genetic differentiation of *M. tuberculosis* isolates (33, 38). Because Beijing family strains are prevalent in many developing areas of Asia, where resources are definitely limited, MIRU-VNTR typing would be the best choice here to study the molecular epidemiology of *M. tuberculosis* strains.

Because TB, especially drug-resistant TB, constitutes a heavy burden in Heilongjiang Province, it is important to understand the possible spreading mechanisms of *M. tuberculosis* and particularly drug-resistant TB. In the present study, we conducted the genotyping of *M. tuberculosis* isolates to determine the prevalence of Beijing family strains and the genotypes of *M. tuberculosis* isolates in Heilongjiang Province using MIRU-VNTR analysis. The appropriate VNTR locus combination for genotyping the *M. tuberculosis* isolates in our area was also evaluated and optimized.

MATERIALS AND METHODS

Mycobacterial specimens. The study included 200 *M. tuberculosis* samples isolated between May 2007 and October 2008 from 200 patients from various regions of Heilongjiang Province who had been diagnosed with pulmonary TB in the Harbin Chest Hospital. All the patients were HIV-1 negative. The average age of the patients was 49.1 years, and 71.5% (143/200) were male. *M. tuberculosis* H37Rv was used as the reference strain.

DNA extraction and molecular identification of *M. tuberculosis* isolates and RD105 deletion detection. Mycobacterial cultures were obtained from clinical specimens after incubation in a BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 Automated System (Becton Dickinson). The cultures were inactivated by 70% ethanol for 2 h (9). DNA was extracted using lysozyme and the phenol-chloroform method (46). Molecular identification of the mycobacterial isolates was performed based on PCR amplification of the 16S rRNA gene and the Rv0577, Rv2073c, and Rv3120 genes (15). Rv2073c amplification was modified as follows: PrimeSTAR HS DNA polymerase (TakaRa, China) was used instead of *Taq* polymerase, and 2 μl of dimethyl sulfoxide was added to ensure good amplification. The amplification cycle was 5 min at 98°C followed by 25 cycles of 1 min at 98°C, 5 s at 65°C, and 40 s at 72°C, with a final 10 min at 72°C. The identification of genomic deletions in region of difference 105 (RD105) was performed by PCR as previously described (14, 26). Briefly, each PCR mixture was prepared in a volume of 20 μl containing 50 ng of DNA, 0.25 U of *Taq* polymerase (TakaRa, China), a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), and 0.2 μM (each) primer. The amplification cycle was 10 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final step for 10 min at 72°C.

Spoligotyping. Spoligotyping of the isolates was performed as described by Kamerbeek et al. (22). The direct repeat (DR) region was amplified with the primer pair, and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer, which were covalently bound to a mem-

TABLE 2. Allelic diversity of different MIRU-VNTR loci[a]

MIRU-VNTR locus	No. of alleles (range)		h	
	Isolate set 1	Isolate set 2	Isolate set 1	Isolate set 2
QUB26	7 (4–10)	8 (3–10)	0.706	0.581
QUB11b	6 (3–8)	9 (0–8)	0.644	0.730
MIRU31	6 (2–7)	7 (2–8)	0.599	0.500
MIRU16	4 (2–6)	4 (2–6)	0.520	0.230
MIRU4	5 (1–5)	6 (0–5)	0.517	0.260
MIRU26	5 (4–9)	8 (3–10)	0.467	0.649
Mtub4	4 (2–5)	6 (0–5)	0.413	0.463
MIRU40	5 (1–5)	6 (1–6)	0.373	0.358
Mtub21	6 (1–8)	9 (1–9)	0.307	0.493
Mtub39	4 (1–6)	6 (1–6)	0.229	0.243
MIRU10	4 (1–4)	4 (1–4)	0.229	0.300
Mtub30	4 (2–5)	4 (2–5)	0.191	0.267
Mtub29	3 (3–5)	5 (2–6)	0.189	0.138
MIRU39	3 (1–3)	4 (1–4)	0.149	0.388
ETR A	2 (3–4)	7 (0–6)	0.146	0.329
QUB4156	2 (2–4)	5 (0–4)	0.107	0.182
ETR C	3 (2–5)		0.068	
ETR B	2 (1–2)		0.066	
MIRU23	1 (5)		0	

[a] Set 1 consists of 44 isolates obtained from May 2007 to November 2007. Set 2 consists of 200 isolates obtained from May 2007 to October 2008.

TABLE 3. The cumulative HGDI with successive addition of each MIRU-VNTR locus

Locus combination[a]	VNTR locus (*h*)[b]	No. of patterns	No. of clusters	No. of clustered isolates	No. of isolates in each cluster	Clustering rate (%)	HGDI (cumulative)
1	QUB11b (0.730)						
2	MIRU26 (0.649)	35	19	184	2–41	82.5	0.9042
3	QUB26 (0.581)	84	33	149	2–23	58.0	0.9686
4	MIRU31 (0.500)	112	33	121	2–18	44.0	0.9808
5	Mtub21 (0.493)	126	33	107	2–16	37.0	0.9867
6	Mtub4 (0.463)	135	30	95	2–15	32.5	0.9888
7	MIRU39 (0.388)	150	23	73	2–14	25.0	0.9913
8	MIRU40 (0.358)	159	18	59	2–12	20.5	0.9935
9	ETR A (0.329)	164	16	52	2–12	18.0	0.9943
10	MIRU10 (0.300)	169	14	45	2–12	15.5	0.9950
11	Mtub30 (0.267)	169	14	45	2–12	15.5	0.9950
12	MIRU4 (0.260)	176	12	36	2–10	12.0	0.9967
13	Mtub39 (0.243)	178	10	32	2–10	11.0	0.9968
14	MIRU16 (0.230)	179	11	32	2–8	10.5	0.9976
15	QUB4156 (0.182)	180	10	30	2–8	10.0	0.9977
16	Mtub29 (0.138)	180	10	30	2–8	10.0	0.9977

[a] The successive addition of each VNTR locus.
[b] The *h* value represents the diversity determined from the 200 isolates.

brane (22). Spoligotypes in binary format were compared with the SpolDB4 database, and the spoligotype international type (SIT) numbers and the clades were also determined (4).

MIRU-VNTR typing. To identify a suitable MIRU-VNTR locus set for genotyping *M. tuberculosis* isolates in this area, 19 loci were selected for analyzing the first set of 44 *M. tuberculosis* isolates (38). The PCR mixture and conditions were the same those for the RD105 deletion identification described above. Genomic DNA of the H37Rv strain and sterile distilled water were used as the positive and negative controls, respectively. PCR products were analyzed on a 1.5% agarose gel against a 100-bp DNA ladder (TakaRa, China), and the copy number at each locus was calculated using a Quantity 1 gel imaging system (Tanon, China). The MIRU-VNTR allelic diversity (*h*) at a given locus was calculated as follows: $h = 1 - \Sigma x_i^2[n/(n-1)]$, where x_i is the frequency of the *i*th allele at the locus, and *n* is the number of isolates (35). The discrimination of the locus combination was calculated using the Hunter-Gaston discriminatory index (HDGI) (16):

$$\mathrm{HGDI} = 1 - \frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)$$

where *N* is the total number of isolates in the typing method, *s* is the number of distinct patterns discriminated by MIRU-VNTR, and n_j is the number of isolates belonging to the *j*th pattern.

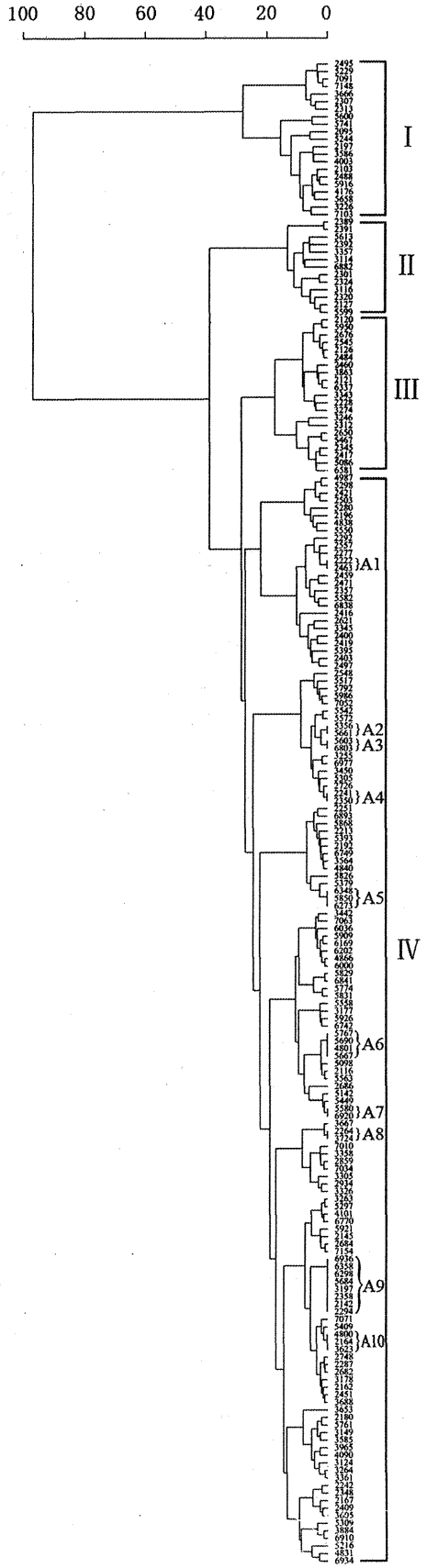
Phylogenetic and cluster analysis. We used the R software, version 2.11.1 (http://cran.r-project.org), for phylogenetic and cluster analysis. A dendrogram was produced from the MIRU-VNTR genotypes of the 200 *M. tuberculosis* isolates. First, the repeat numbers of MIRU-VNTR genotypes were standardized based on a *z*-score normalization. Then, a similarity coefficient matrix of the *M. tuberculosis* isolates was obtained by calculating the Euclidean distances between isolates from the standardized data. Finally, clustering was performed, and a phylogenetic tree was constructed using Ward's parameter with the matrix. The *M. tuberculosis* isolates analyzed in this study were classified into two groups, characterized by clustered and nonclustered *M. tuberculosis* isolates. A molecular cluster was defined as two or more *M. tuberculosis* isolates having identical genetic patterns as determined by MIRU-VNTR genotyping. The isolates with unmatched genetic profiles were considered nonclustered strains. Assuming that one patient from each cluster corresponded to the index case at the origin of infection, the clustering rate was calculated using the following formula: clustering rate = $(n_c - c)/n$, where n_c is the total number of clustered isolates, *c* is the number of isolate clusters, and *n* is the total number of isolates in the sample (37).

Statistical analysis. Associations among multiple categorical variables were assessed using R, version 2.11.1, by a chi-square test or Fisher's exact test when the theoretical frequency was less than five. Two-by-two tables were assessed by a chi-square test (here, Yates' continuity correction was needed when the value was less than five), and results were expressed as odds ratios (OR) with 95% confidence intervals (95% CI). The agreement between spoligotyping and RD105 deletion typing was assessed using kappa statistics; the agreement was considered good for values of kappa above 0.75. *P* values of <0.05 were considered statistically significant.

RESULTS

Epidemic of Beijing family strains in Heilongjiang Province. During the study period, 200 *M. tuberculosis* isolates identified using both the BACTEC 960 automated system and molecular methods were collected. First, we analyzed the correlation between spoligotyping and RD105 deletion for the identification of the Beijing genotype using 44 isolates collected from May 2007 to November 2007. Among the 44 *M. tuberculosis* isolates, spoligotypes of 41 isolates were classified into three designated SITs according to the SpolDB4 database (Table 1). Among these, 40 isolates were Beijing family strains. The most frequent genotype (39/41) was the typical Beijing spoligotype SIT1, which has only spacers 35 to 43; the only other Beijing genotype belonged to spoligotype SIT190. One isolate, with an SIT number of 1793, was not designated in the database. The remaining three isolates showed new spoligotypes, which were not registered in SpolDB4 database. Interestingly, one isolate (2460) showed a unique genotype with only two spacers, 35 and 36. We found that 40 isolates lacking RD105 exhibited Beijing family spoligotypes (Table 1). The *M. tuberculosis* isolate 2460 also lacked RD105. The results of the kappa statistics analysis showed that the agreement of spoligotyping and RD105 deletion detection in identifying the Beijing family genotype was high ($\kappa = 0.8451$). Subsequently, instead of using spoligotyping, RD105 deletions in the other 156 *M. tuberculosis* strains were examined, and we found that 179 of the 200 isolates (89.5%) had the Beijing family genotype, while 21 (10.5%) were non-Beijing family strains.

Optimal combination of MIRU-VNTR loci for genotyping *M. tuberculosis* isolates in Heilongjiang Province. First, to evaluate and determine the most suitable loci for genotyping the *M. tuberculosis* isolates epidemic in Heilongjiang Province, we analyzed 19 MIRU-VNTR loci, which had been previously identified as a suitable locus combination for genotyping *M. tuberculosis* isolates in the regions where the Beijing family is



dominant (19, 27) (Table 2). The allelic diversity (h) of the first set of 44 *M. tuberculosis* isolates at each MIRU-VNTR locus varied significantly. Among the 19 loci, the allelic diversity for 2 loci (QUB11b and QUB26) exceeded 0.6, suggesting that they are highly discriminating (30). Seven loci (MIRU4, MIRU16, MIRU26, MIRU31, MIRU40, Mtub21, and Mtub4) showed moderate discrimination ($0.3 \leq h \leq 0.6$), but ETR C ($h = 0.068$) and ETR B ($h = 0.066$) were less polymorphic. Diversity was not observed for the MIRU23 locus ($h = 0$). Thus, the loci ETR C, ETR B, and MIRU23, having discriminatory powers of less than 0.1, were excluded from the subsequent MIRU-VNTR analysis.

Next, we analyzed the 200 *M. tuberculosis* isolates collected from May 2007 to October 2008 using the remaining 16 MIRU-VNTR loci. All 16 loci displayed an allelic diversity similar to the original 19 loci (Table 2). The highest diversity among the 200 isolates was observed at QUB11b ($h = 0.730$), and the lowest diversity was observed at Mtub29 ($h = 0.138$). The HGDI of the 16-locus set was as high as 0.9977. However, because the 16-locus procedure still did not meet the requirements of cost and labor expenditure for high-throughput genotyping, we then tried to optimize the locus combination while minimizing the number of loci. Based on the allelic diversity of each MIRU-VNTR locus, the cumulative HGDI of the locus combination by successive addition of a locus was compared (Table 3). The cumulative HGDI and clustering rate of the 10-locus set were equal to that of the 11-locus set (HGDI, 0.9950; clustering rate, 15.5%); they were also the same for the 15- and the 16-locus sets (HGDI, 0.9977; clustering rate, 10.0%). The set of the first seven loci with the highest allelic diversity gave an HGDI of 0.9913 and a clustering rate of 25.0%.

VNTR profiles and genotypes of the *M. tuberculosis* isolates in Heilongjiang Province. The MIRU-VNTR genotyping results showed that the 200 isolates were classified into 180 genotypes. A total of 170 isolates had unique patterns, while the remaining 30 isolates were in 10 clusters. A dendrogram was constructed based on the genotypes of 200 isolates using 16 loci (Fig. 1). The isolates were divided into four groups based on phylogenetic clustering and genotypic characteristics. Groups I to IV contained 21, 13, 21, and 145 isolates, respectively. Among the 179 Beijing family isolates, 144 (99.3%) were in group IV; the remaining 35 isolates were in groups I, II, and III ($P < 0.0001$), and all the clustered isolates were in group IV ($P = 0.0018$), suggesting that the distributions of the Beijing family isolates and clustered isolates were distinctive among the four groups (Table 4).

Characteristics of the clustered isolates. Thirty Beijing family isolates (30/179, or 16.8% of the Beijing family strains) were determined to be in 10 clusters (A1 to A10), with the clustering rate of 10.0% based on 15-locus MIRU-VNTR patterns. In contrast, none of 21 non-Beijing family isolates were clustered (OR, 0; 95% CI, 0 to 1.024; $P = 0.087$) (Table 5). Most of the

FIG. 1. Dendrogram of 200 *M. tuberculosis* isolates from Heilongjiang Province. The phylogenetic tree was produced from the MIRU-VNTR genotypes which were derived from 16 of the 19 loci by excluding ETR B, ETR C, and MIRU23. A1 to A10, cluster names.

TABLE 4. Differences of *M. tuberculosis* characteristics among the four subgroups

Isolate characteristic	Total no. of isolates	No. (%) of isolates by subgroup[a]				*P* value[b]
		I (n = 21)	II (n = 13)	III (n = 21)	IV (n = 145)	
Resistance						
Streptomycin	85	9 (42.9)	6 (46.2)	8 (38.1)	62 (42.8)	0.9704*
Isoniazid	92	9 (42.9)	8 (61.5)	7 (33.3)	68 (46.9)	0.4317*
Rifampin	55	4 (19.0)	7 (53.8)	3 (14.3)	41 (28.3)	0.0815
Ethambutol	48	4 (19.0)	6 (46.2)	3 (14.3)	35 (24.1)	0.2070
MDR	51	4 (19.0)	6 (46.2)	2 (9.5)	39 (26.9)	0.1032
Four-drug susceptibility	77	8 (38.1)	3 (23.1)	9 (42.9)	57 (39.3)	0.6786*
Four-drug resistance	23	2 (9.5)	3 (23.1)	1 (4.8)	17 (11.7)	0.4402
Obtained from a patient with acquired TB	126	17 (81.0)	9 (69.2)	11 (52.4)	89 (61.4)	0.2294
Obtained from a patient with hemoptysis	169	15 (71.4)	9 (69.2)	20 (95.2)	125 (86.2)	0.0562
Beijing strain	179	3 (14.3)	12 (92.3)	20 (95.2)	144 (99.3)	<0.0001
Clustered	30	0 (0)	0 (0)	0 (0)	30 (20.7)	0.0018

[a] n, number of isolates in the subgroup.
[b] Values marked with an asterisk were determined by a chi-square test; other values were determined by a Fisher's exact test.

clusters were small: six (A1 to A4, A7, and A8) contained only two members; two (A5 and A10) contained three members; cluster A6 contained four members. The largest cluster, A9, contained eight members. In addition, the clustering rates of the two periods, May 2007 to May 2008 (106 isolates) and June 2008 to October 2008 (94 isolates), were 6.4% and 12.8%, respectively (OR, 0.3240; 95% CI, 0.1161 to 0.8265; P = 0.0088).

To determine if there was any correlation between the clustering characteristics and the geographical origins of the isolates, we investigated the home addresses of the patients in the clusters from the available medical records. We found that the isolates belonging to clusters A2 and A7 were scattered throughout the Heilongjiang Province while clusters A3 to A6, A9, and A10 were registered in Harbin City.

Drug susceptibility patterns of the *M. tuberculosis* isolates in Heilongjiang Province. To determine the association between drug resistance patterns and genotypic characteristics, drug susceptibility to the four first-line antituberculosis drugs, i.e., streptomycin, isoniazid, rifampin, and ethambutol, was examined using an automated BACTEC MGIT 960 SIRE system (Becton Dickinson). A total of 77 isolates (38.5%) were susceptible to all four drugs; 123 (61.5%) were resistant to at least one drug, and 51 (41.5%) were MDR *M. tuberculosis* (Tables 4 and 5). The drug susceptibility patterns of the isolates among the four genotype groups were not significantly different (Table 4). Of the 51 MDR *M. tuberculosis* isolates, 48 isolates were found to be the Beijing family strains, and 3 were non-Beijing family strains. Of the Beijing family strains, 26.8% (48/179) were MDR, and 14.3% (3/21) of the non-Beijing family strains were MDR. The rates of MDR *M. tuberculosis* among Beijing and non-Beijing family strains were not statistically different (OR, 0.4564; 95% CI, 0.0824 to 1.6670; P = 0.2127). Resistance to at least one drug was observed more frequently among Beijing family strains (63.1%, or 113/179) than among non-Beijing family strains (47.6%, or 10/21), but the difference was not statistically significant (OR, 1.8771; 95% CI, 0.6828 to 5.2244; P = 0.1670) (Table 5).

DISCUSSION

The Beijing family strains currently prevail throughout China. RD105 deletion has recently been reported to serve as a genetic marker for Beijing family strains (44), and several

TABLE 5. Differences of *M. tuberculosis* characteristics between Beijing and non-Beijing family

Isolate characteristic	Total no. of isolates	No. (%) of isolates[a]		OR	95% CI	*P* value[b]
		Beijing (n = 179)	Non-Beijing (n = 21)			
Resistance						
Streptomycin	85	78 (43.6)	7 (33.3)	0.6488	0.2110–1.8176	0.3691
Isoniazid	92	84 (46.9)	8 (38.1)	0.6972	0.2381–1.9189	0.4423
Rifampin	55	52 (29.1)	3 (14.3)	0.4086	0.0739–1.4876	0.1517
Ethambutol	48	45 (25.1)	3 (14.3)	0.4978	0.0898–1.8235	0.4056*
MDR	51	48 (26.8)	3 (14.3)	0.4564	0.0824–1.6670	0.2127
Four-drug susceptibility	77	66 (36.8)	11 (52.4)	1.8771	0.6828–5.2244	0.1670
Four-drug resistance	23	21 (11.7)	2 (9.5)	0.7928	0.0837–3.6902	0.9510*
Obtained from a patient with acquired TB	126	109 (60.9)	17 (90.0)	2.7174	0.8390–11.5638	0.0717
Obtained from a patient with hemoptysis	169	153 (85.5)	16 (76.2)	1.8323	0.4830–5.8459	0.4275*
Clustered	30	30 (16.8)	0 (0)	0	0–1.0241	0.0869*

[a] n, number of isolates in the group.
[b] Values marked with an asterisk were determined by a continuity-adjusted chi-square test; other values were determined by a chi-square test.

studies have used this method to identify them (6, 26, 45). It is financially economical, labor saving, and especially suitable for high-throughput analysis. In this study, we found good agreement between RD105 deletion detection and spoligotyping. One strain (2460) showed a novel spoligotype containing only spacers 35 and 36 and an RD105 deletion. According to the definition of the Beijing family spoligotype, these strains contain at least three spacers among direct repeats 35 to 43; however, strain 2460 can be included in the Beijing family because it lacks RD105.

We found that 89.5% of the *M. tuberculosis* isolates in Heilongjiang Province were Beijing family strains. This genotype accounts for 80 to 90% of the *M. tuberculosis* strains currently epidemic in the Beijing area (19); it is also prevalent in Ningxia (67%), Shanghai (89%), Zhejiang (70%), Tianjin (91.7%), and Guangxi (55.3%) but less prevalent in Guangdong (25%) (5, 24, 25, 36, 48). Hence, Heilongjiang Province is one of the regions where the proportion of the Beijing genotype is the highest. This genotype is thought to be associated with drug resistance (1, 10, 23, 41). However, less association has been reported in other geographic settings (2, 3, 20, 43). In the present study, the statistical analysis showed that there was no difference between the Beijing and non-Beijing genotype strains in drug resistance patterns, indicating that the Beijing genotype is less likely to be associated with the high prevalence of drug resistance and *M. tuberculosis* TB in our area.

Molecular typing by MIRU-VNTR has been used in epidemiology studies, and its stability is adequate for tracking recent transmission and distinguishing relapses and reinfections (39). Currently, the system based on 12 loci (29) is most widely used among the different sets of MIRU-VNTR loci. However, it is not effective for the analysis of clustered isolates (7). Other sets of MIRU-VNTR loci, such as the 14-locus set and the 15-locus set, have improved the discrimination of unrelated isolates (23, 38). An optimized set of 24 loci has also been defined; however, not all 24 loci are required for genotyping *M. tuberculosis* strains in any given situation (38) as the number of loci required depends on the lineage known to be prevalent in the investigated area.

In the present study, we found that the 16 of the 19 loci had high discriminatory diversity. This 16-locus set showed strong discriminatory power in analyzing the *M. tuberculosis* strains in our area (HGDI of 0.9977). Because the ability of the different locus combinations to differentiate the *M. tuberculosis* strains varied, we evaluated various sets of MIRU-VNTR loci to identify a minimal subset that provided discrimination comparable to that of the 16 loci. We found that the locus Mtub29 could be excluded from the set because the HGDI and clustering rate of the remaining 15 loci were the same as those of the 16 loci. The HGDI and the clustering rate of a 10-locus set were comparable to those of the 16-locus set. Therefore, we suggest that this 10-locus set be used as a first-line set for genotyping *M. tuberculosis* isolates in Heilongjiang Province, especially for routine epidemiological investigation and large-scale genotyping. Comparing the HGDI and the clustering rate of this locus set with those of various locus sets reported in other areas of China, we found that the discriminatory power of the 15-locus set used in the present study was the highest and that the clustering rate was the lowest (Table 6).

However, MIRU-VNTR loci showed variation in the ability to differentiate Beijing genotype strains from different geographical areas. Trying to explore the loci showing high discriminatory power among Beijing genotype strains in various areas of the world (Table 7), we found that at least 11 and 14 loci showed high enough diversity among the locally circulating Beijing genotype strains in China and Japan, respectively. Therefore, we recommend them as the predominant candidates (Table 7, underlined median *h* value for China and Japan). Since the Beijing genotype is dominant in China and Japan, we also suggest taking the 14 loci that show high diversity among the strains epidemic in the two countries as the predominant candidates for Asia (Table 7, underlined Asian median values). Meanwhile, the loci showing very low diversity (Table 7, boldface), 11 from China and 9 from Japan, may not need to be included for future studies. However, there are still some loci that showed high variation in differentiating Beijing genotype strains. For example, the loci MIRU10 and MIRU16 showed moderate diversity in Hong Kong and Gansu but low diversity in the other areas of China. The locus VNTR4120 was highly discriminatory in Japan (*h* of 0.902) but less discriminatory in China (*h* of 0.092).

TABLE 6. Discriminatory index of different locus sets used in various regions of China and the clustering rates

Area	Locus set	Clustering rate (%)	HGDI	Reference
Hong Kong	17 loci	17.4	0.9900	23
Shanghai	16 loci	16.1	0.9982	52
	7 loci	25.0	0.9957	
Beijing	24 loci	15.3	0.9920	19
	15 loci	18.1	0.9900	
	12 loci	59.7	0.7880	
Fujian	12 loci	17.1	0.9808	18
Gansu	15 loci	42.1		42
Zhejiang	15 loci	30.0	0.9905	49
Eight regions	12 loci	33.5	0.9780	13
Five regions	19 loci	15.7	0.9949	27
Heilongjiang	15 loci	10.0	0.9977	This study
	10 loci	15.5	0.9950	
	7 loci	25.0	0.9913	

In Japan, most of the loci reported showed comparatively high discriminatory power; therefore, considering labor and cost, some loci with moderate *h* values (>0.3) may not need to be included. Russia is much different from Japan and China in allelic diversity of the MIRU-VNTR loci, and the *h* values of most loci are much lower than those in China and Japan. This difference may imply that the loci which are suitable for genotyping the isolates epidemic in Asia may not be suitable for genotyping isolates in Russia.

Active transmission of drug-resistant *M. tuberculosis* strains in a community is an emerging problem. It is generally assumed that the proportion of clustered strains in a population reflects the level of active transmission (11, 28). The present study using 15 loci showed that the clustering rate in Heilongjiang Province is 10.0%, which is lower than the rates reported in other areas (Table 6). Though some loci that show moderate or high discriminatory power in other areas were not included in the present study, omitting them will not increase the clustering rate in this area because the loci may decrease the clustering trend of the strains by decreasing the diversity. The

TABLE 7. Allelic diversity of different MIRU-VNTR loci for differentiating *M. tuberculosis* Beijing family strains in different areas

Locus	Allelic diversity (h) by region[a]														
	Russia[b]			Japan[c]				China[d]							Median for Asia
	St. Petersburg (n = 48)	West Siberia (n = 51)	Median	Kobe (n = 181)	Japan (n = 240)	Chiba (n = 185)	Median	Beijing (n = 72)	Shanghai (n = 189)[e]	Hong Kong group 1 (n = 51)	Hong Kong group 2 (n = 243)	Gansu (n = 202)	Heilongjiang (n = 179)	Median	
VNTR4120	0.370		0.370	0.902	0.902	0.882	0.902		0.092*					0.092	0.892
QUB3232	0.729		0.729	0.880	0.909	0.813	0.880				0.804			0.804	0.847
VNTR3820	0.542		0.542	0.800	0.871	0.817	0.817		0.821					0.821	0.819
QUB11b	0.205	0.210	0.208	0.772	0.815	0.763	0.772	0.651	0.689	0.618	0.669		0.704	0.669	0.697
QUB18		0.740	0.740			0.629	0.629		0.607	0.740	0.488			0.607	0.618
Mtub24					0.591	0.614	0.603		0.223					0.223	0.591
QUB26	0.636	0.780	0.708	0.741	0.764	0.215	0.741	0.518	0.630	0.299	0.314		0.607	0.518	0.563
Mtub21	0.330	0.110	0.220	0.393	0.598	0.537	0.537	0.556	0.544			0.690	0.396	0.550	0.544
QUB11a				0.685	0.752	0.535	0.685		0.538	0.384	0.514			0.514	0.537
QUB3336				0.487	0.642	0.482	0.487				0.214			0.214	0.485
QUB4156	0.082		0.082	0.611	0.623	0.603	0.611	0.395	0.469		0.167		0.182	0.289	0.469
Mtub4	0.000		0.000	0.459	0.468	0.581	0.468	0.306	0.266				0.391	0.306	0.425
MIRU26	0.520		0.520	0.383	0.314	0.283	0.314	0.353	0.614	0.200		0.560	0.596	0.560	0.368
QUB1895				0.364	0.337	0.468	0.364		0.365	0.229	0.206			0.229	0.351
VNTR2372					0.595	0.345	0.470		0.177*					0.177	0.345
QUB15					0.537	0.629	0.583		0.032*		0.132			0.082	0.335
MIRU31	0.160	0.000	0.080	0.322	0.270	0.379	0.322	0.169	0.328		0.156	0.370	0.395	0.328	0.325
ETR F					0.237	0.499	0.368		0.290					0.290	0.290
MIRU10	0.082		0.082	0.419	0.431	0.291	0.419	0.144	0.239	0.377		0.160	0.154	0.160	0.265
MIRU40	0.122	0.390	0.256	0.327	0.229	0.473	0.327	0.194	0.147*	0.196		0.350	0.292	0.196	0.261
MIRU16	0.082		0.082	0.310	0.258	0.421	0.310	0.068	0.131	0.058		0.580	0.200	0.131	0.229
ETR A	0.158	0.000	0.079	0.147	0.223	0.165	0.165	0.232	0.031*	0.201	0.188	0.280	0.238	0.217	0.201
Mtub39	0.000	0.000	**0.000**	0.186	0.215	0.271	0.215	0.171	0.061*			0.120	0.174	0.146	0.174
MIRU39	0.000		**0.000**	0.221	0.156	0.160	0.160	0.119	0.141	0.320	0.040	0.100	0.290	0.141	0.158
Mtub30	0.042		0.042	0.403	0.379	0.210	0.379	0.068	0.091*			0.090	0.133	**0.090**	0.133
Mtub29	0.087	0.180	0.134	0.043	0.095	0.103	**0.095**	0.119	0.061*				0.123	**0.119**	**0.103**
MIRU23	0.000	0.000	**0.000**	0.176	0.158	0.124	0.158	0.014	0.061*			0.030		**0.030**	**0.093**
QUB5(MIRU27)	0.000		**0.000**	0.115	0.081	0.074	**0.081**	0.014	0.031*			0.100		**0.031**	**0.078**
MIRU4	0.000		**0.000**	0.086	0.049	0.000	**0.049**	0.120	0.061	0.019	0.072		0.212	**0.061**	**0.067**
MIRU20	0.120		**0.120**	0.022	0.065	0.063	**0.063**	0.014	0.061*					**0.038**	**0.061**
ETR C	0.042	0.000	**0.021**	0.022	0.057	0.063	**0.057**	0.094		0.165	0.057	0.000		**0.076**	**0.057**
Mtub34	0.000		**0.000**	0.066	0.033	0.000	**0.033**	0.014	0.089*					**0.052**	**0.033**
QUB23					0.025		0.025				0.016			0.016	0.021
ETR B	0.000		**0.000**	0.033	0.017	0.032	**0.032**	0.014	0.000*	0.000	0.064	0.020		**0.014**	**0.019**
VNTR0569						0.011	0.011		0.000*					0.000	0.006
QUB1451				0.033			0.033		0.000*		0.008			0.004	0.008
MIRU2	0.000		**0.000**	0.000	0.008	0.000	**0.000**	0.000	0.000*					**0.000**	**0.000**
MIRU24	0.000		**0.000**	0.000	0.000	0.042	**0.000**	0.000	0.000*					**0.000**	**0.000**
ETR E		0.000	**0.000**												0.000

[a] n, number of isolates; underlining, corresponding locus was recommended; boldface, corresponding locus was not recommended.
[b] See the following references: for St. Petersburg, 31; for West Siberia, 40.
[c] See the following references: for Kobe, 17; for Japan, 32; for Chiba, 51. The Japan strains were from a drug resistance survey in Japan in 2002.
[d] See the following references: for Beijing, 19; for Shanghai, 52; for Hong Kong group 1, 23; for Hong Kong group 2, 21; and for Gansu, 42. Hong Kong strains were collected in 2001 (group 1) or 2001 to 2003 (group 2).
[e] For values marked with an asterisk, the number of samples was 65.

comparatively low rate and the small size of the clusters suggest that the high resistance of *M. tuberculosis* in Heilongjiang Province is not related to recent transmission but, rather, may be related to reactivation or inappropriate therapy. However, the clustering rate is still increasing and was much higher in late 2008 (12.8%) than in 2007 (6.4%), suggesting that more effective control strategies are needed.

This is the first report describing the molecular epidemiology of *M. tuberculosis* isolated from patients with pulmonary TB in Heilongjiang Province, China. The low clustering rate in our area indicates that only mild active transmission occurred in the time period studied. We defined the most suitable MIRU-VNTR locus set for analyzing the *M. tuberculosis* isolates in Heilongjiang Province, where Beijing family strains are prevalent. In our hands, the 15-locus set provided a high degree of discrimination; the 10-locus set was shown to be ideal for use in first-line molecular typing in future research although we still need to examine the discriminatory power of the rest of the recommended loci.

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ORIGINAL ARTICLE

Drug resistance and IS*6110*-RFLP patterns of *Mycobacterium tuberculosis* in patients with recurrent tuberculosis in northern Thailand

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ABSTRACT

The emergence of drug resistant *Mycobacterium tuberculosis* has become a global threat to tuberculosis (TB) prevention and control efforts. This study aimed to determine the drug resistance profiles and DNA fingerprints of *M. tuberculosis* strains isolated from patients with relapsed or retreatment pulmonary TB in Chiang Rai province in northern Thailand. Significant differences in multidrug resistance (MDR) ($P = 0.025$) and resistance to isoniazid ($P = 0.025$) and rifampin ($P = 0.046$) between first and second registrations of patients with retreatment TB were found. However, there were no significant differences in resistance to any drugs in patients with relapsed TB. The rate of MDR-TB strains was 12.2% among new patients at first registration, 22.5% among patients with recurrence who had previously undergone treatment at second registration and 12.5% at third registration. Two retreatment patients whose initial treatment had failed had developed MDR-TB with resistance to all TB drugs tested, including rifampin, isoniazid, streptomycin and ethambutol. IS*6110*-RFLP analysis revealed that 66.7% (10/15 isolates) of MDR-TB belonged to the Beijing family. In most cases, IS*6110*-RFLP patterns of isolates from the same patients were identical in relapse and retreatment groups. However, some pairs of isolates from retreatment patients after treatment failure had non-identical IS*6110*-RFLP patterns. These results suggest that, after failure and default treatment, patients with retreatment tuberculosis have a significantly greater risk of MDR-TB, isoniazid and rifampin resistance than do other patients.

Key words drug resistance, IS*6110*- restriction fragment length polymorphism patterns, recurrent tuberculosis.

Tuberculosis remains a major public health problem worldwide. Almost two million people die of TB annually and an estimated one-third of the world's population has latent infection. The situation is worse in developing countries in South-East Asia and Africa where MDR is on the increase. In six Asian countries, drug resistance is reportedly present in an estimated 2.8% of new and 18.8% of previously treated TB patients (1). Thailand, which is ranked 18th on a list of the 22 countries with the largest TB burdens, had a prevalence of approximately 192/100,000

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List of Abbreviations: BCG, bacillus Calmette–Guérin; EMB, ethambutol; INH, isoniazid; IS*6110*, insertion sequence *6110*; *M. tuberculosis*, *Mycobacterium tuberculosis;* MDR, multi-drug resistance; MDR-TB, multi-drug resistance tuberculosis; PZA, pyrazinamide; RFLP, restriction fragment length polymorphism; RMP, rifampin; SM, streptomycin; TB, tuberculosis; VNTR, variable number tandem repeat; XDR, extensive drug resistance.

people for all forms and an incidence rate of 62 new smear-positive cases per 100,000 in 2007 (2). The first national drug-resistance survey conducted in 2002 reported 1% of MDR-TB in new TB cases and 20% in previously treated cases (3). The rates had increased to 1.7% and 34.5%, respectively, by the second survey in 2006 (3). Moreover, TB surveillance during 1996–1998 showed that a high proportion of TB cases with drug resistance occurred in Chiang Rai province in northern Thailand.

In general, patients who have undergone successful treatment with anti-TB drugs can develop active disease again subsequently, referred to as recurrent TB. However, the pathogenesis of recurrence and classification of recurrent cases are still unclear (4). Recurrence of TB may be a result of regrowth of the treated bacterial strain in patients otherwise previously treated successfully, or of re-infection (4). Relapse refers to a patient becoming culture-positive again, or evidencing clinical or radiographic deterioration consistent with active TB, sometime after completion of apparently successful anti-TB drug therapy that had resulted in culture-negativity (5). The term retreatment refers to patients with recurrent TB who defaulted before completing their previous therapy or in whom initial treatments failed (4). Whether recurrent TB represents exogenous re-infection by a new strain of *M. tuberculosis* or endogenous reactivation of the original strain is controversial and has been debated for decades (6, 7). The importance of each of these possibilities likely varies according to the epidemiological context, the spread of MDR-TB, HIV infection (8), and the immigration of people from developing countries, which could modify disease transmission in areas at low risk of TB (8). In patients infected with HIV and MDR-TB there is evidence for a greater risk of reactivation than of re-infection (9, 10).

The most-widely used method for typing *M. tuberculosis* to determine whether recurrent TB represents endogenous reactivation or exogenous re-infection has been a molecular method involving Southern blotting of *Pvu*II-digested chromosomal DNA and hybridization with the insertion sequence (IS) *6110* (11). In practice, it is generally accepted that two or more isolates with identical or near-identical (± one band) IS*6110* fingerprints (known as clusters) represent a recent transmission event (12). This technique is thus useful in distinguishing between recent epidemiological events (transmission) and distant epidemiological events (reactivation) (12). In a single TB patient with a TB-free interval, it is assumed that isolates with identical IS*6110*- RFLP patterns denote endogenous reactivation of the previously infecting bacteria (13).

Apart from differentiating between endogenous reactivation and exogenous re-infections, IS*6110*-RFLP has allowed identification of different *M. tuberculosis* strains with varying degrees of virulence and drug resistance in different geographical areas (14). Up to now, the largest family of *M. tuberculosis* strain has been the Beijing family. The highest prevalence of this family reportedly occurs in Asian patients (15) and it is associated with various phenotypes such as drug-resistance (14), treatment failure, relapse and febrile response to TB treatment (16). In several Asian studies, the proportion of TB due to Beijing strains has been > 50% (14). However, because the IS*6110*-RFLP patterns vary between different geographical areas, there is so far limited available data regarding recurrent TB in Thailand. Although there was a national anti-TB drug resistance survey during 1997–1998 as part of a global project to evaluate IS*6110*-RFLP patterns and the extent of clustering, this study did not assess linkage to TB treatment history and development of drug resistance. Other subsequent study in Chiang Rai assessed acquired drug resistance in patients who had become positive again after completion, default from or failure of a standardized treatment regimen (10). These researchers commonly found non-identical IS*6110*-RFLPs in the first and subsequent episodes in TB-HIV patients. Successful treatment of TB depends upon selection of an effective drug regimen; however, drug resistance can evolve in originally drug-susceptible strains during anti-TB treatment. Therefore, this study aimed to evaluate the relationship between the quality of treatment and development of resistance by assessing drug resistant *M. tuberculosis* in relation to the molecular patterns in recurrent TB patients with either relapse or retreatment TB in Chiang Rai province in northern Thailand.

MATERIALS AND METHODS

Isolates

Two hundred and three *M. tuberculosis* isolates from 77 pulmonary TB patients who had registered twice or more, kindly provided by the Microbiology Laboratory, Chiang Rai provincial hospital and the National TB Reference Laboratory, Bureau of Tuberculosis, Thailand, were cultured. These isolates were selected from isolates of patients with recurrent TB that had been stored as part of a ten year analysis of TB by Chiang Rai provincial hospital from 1 January 1997 to 31 December 2006, as mentioned above. Among these 203 *M. tuberculosis* isolates, only 92 were successfully cultured and subjected to IS*6110*-RFLP analysis. These 92 isolates were from 42 patients with relapse or retreatment TB who had registered twice or more with pulmonary TB and had been treated with anti-TB drug regimens.

Patients

The patients were diagnosed by medical history, chest radiographic findings, microscopic examination for acid-fast bacilli in sputum and positive cultures of *M. tuberculosis*, followed by species identification by biochemical tests and gene probes (ACCUProbe, GenProbe, San Diego, CA, USA) at the National TB Reference Laboratory, Bureau of Tuberculosis, Thailand. The patients were categorized according to World Health Organization criteria (17), which include ascertaining whether or not the patients have previously received TB treatment. The TB drug regimens were based on the recommendations of the National Tuberculosis Program, Ministry of Public Health, Thailand. The standard TB treatment drugs were INH, RMP, PZA and EMB. Because immunocompromised patients are reportedly at greater risk of re-infection TB (10, 18), patients co-infected with HIV were excluded from this study by using particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and micro particle enzyme immunoassay (AxSYM HIV Ag/Ab Combo, Abbott Laboratories, Abbott Park, IL, USA).

This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Reference number 3/ 2550).

Case definitions

According to World Health Organization definitions of cases and treatment outcomes (17), a patient whose sputum smear or culture is positive at the beginning of the treatment but negative in the last month of treatment and on at least one previous occasion is defined as "cured". A patient who completes treatment but does not have negative sputum smears or cultures in the last month of treatment and on at least one previous occasion is defined as "completed". "Treatment success" is the sum of cured and completed. "Failure" is patients whose sputum smears or cultures are positive 5 months or more after commencing treatment. A patient whose treatment is interrupted for two or more consecutive months is defined as "default". "Died" refers to patient who have died for any reason during the course of treatment. "Relapses" refer to new TB episodes occurring after a period without TB. "Retreatments" (after failure or default) are continuations of TB episodes that require changes in treatment regimens: this traditionally requires re-registration.

In TB, drug resistance can be primary or acquired, primary resistance being defined as resistance in patients without a history of previous treatment. Acquired drug resistance is defined as resistance in those who have previously undergone TB treatment. Drug-resistant TB is classified as monodrug (resistance to a single first-line drug), polydrug (resistance to two or more first-line drugs) and multidrug resistance (MDR) (resistant to isoniazid and rifampicin, with or without resistance to any other drugs) (17).

Antibiotic sensitivity testing

Drug susceptibility testing was performed by the fully automated BACTEC MGIT 960 system (Becton Dickinson Biosciences, Sparks, MD, USA) for testing *M. tuberculosis* susceptibility to SM, INH, RMP and EMB at the following final drug concentrations: 1.0 μg/mL for SM, 0.1 μg/mL for INH, 1.0 μg/mL for RMP and 5.0 μg/mL for EMB.

Insertion sequence *6110*-restriction fragment length polymorphism

Insertion sequence *6110*-RFLP analysis was done by Southern blotting and DNA hybridization with an IS*6110* probe (10, 19, 20). Briefly, chromosomal DNA of *M. tuberculosis* was extracted by chloroform-isoamyl alcohol. Three micrograms of DNA were digested with 10 U/μL of *Pvu*II (Boehringer Mannheim, Mannheim, Germany) and electrophoresed in 0.8% agarose. The extracted DNA from *M. tuberculosis* MT14323 strain was used as control marker. DNA fragments were transferred to a nylon filter (Sigma Chemical, Saint Louis, MO, USA) by the capillary method (11) and hybridized with digoxiginin-labelled *Bam*HI-*Sal*I fragment of pDC73 (21). The plasmid pDC73 contains a portion of the insertion sequence IS*6110* on the right side of the *Pvu*II restricted site. The IS*6110* hybridization patterns were analyzed using Gelcompar II version 1.5 (Applied Maths, Kortrijk, Belgium). Based on 78% or more similarity as previously described, the isolates were classified as members of the Beijing family or Nonthaburi group, (20).

Data analyses

The data were statistically analyzed using SPSS version 17.0. Comparison of pair isolates within individuals was performed to assess similarity between patterns. Drug sensitivity test profiles between the first and the subsequent TB registrations were associated by X^2 test. A P value $< p$; 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of patients

The median ages of patients in the relapse and retreatment TB groups were 54 (range 25–74) and 47 (range 35–65) years, respectively. Thirty-four and eight patients had two and three registrations of pulmonary TB, respectively. The

median interval between the first and second registrations was 13 months (range 3–65), with 21 months (range 8–46) for previous cure, 26 (range 7–63) for completed treatment, 8 (range 4–16) for failed treatment and 24 (range 15–42) for default treatment. The median interval between the second and third registrations was 9 months (range 2–38). Of the 42 patients, 28 were male (67.7%) and 14 female (33.3%). Twenty-two patients (52.4%) had relapsed TB after previously successful treatment (cure = 17, completed = 5), and the rest were retreatment TB, 16 (38.1%) after treatment failure and 4 (9.5%) after default treatment. The second treatments resulted in 23 patients (54.8%) having successful treatment (cure = 21, completed = 2), five (11.9%) treatment failures and 9 (21.4%) default treatment. Five patients (11.9%) died during the course of treatment. Eight patients received third treatments. However, treatments outcomes were available for only six of these patients: two with cure, two with failure and two with death (Table 1).

Table 1. Clinical characteristics of 42 patients with recurrent tuberculosis

No.	Age/ Sex (years)	TB treatment outcome			Time interval between	
		First	Second	Third	First–second (months)	Second–third (months)
1	68/F	cure	died	–	46	–
2	62/M	complete	cure	not-available	43	2
3	56/F	cure	cure	–	18	–
4	46/M	cure	default	died	43	19
5	46/M	cure	cure	cure	17	38
6	47/F	cure	cure	–	14	–
7	55/M	complete	complete	–	65	–
8	26/M	cure	cure	–	17	–
9	25/M	complete	default	–	63	–
10	28/F	cure	cure	–	9	–
11	53/F	cure	died	–	19	–
12	56/M	complete	failure	–	12	–
13	46/F	cure	cure	–	16	–
14	27/F	cure	died	–	13	–
15	43/F	cure	cure	–	26	–
16	62/M	complete	died	–	11	–
17	65/M	cure	default	–	23	–
18	67/F	cure	cure	–	14	–
19	38/M	cure	cure	–	11	–
20	74/M	cure	died	–	22	–
21	69/	cure	cure	–	8	–
22	61/M	cure	cure	–	11	–
23	65/M	default	default	failure	42	20
24	40/M	default	default	–	14	–
25	42/M	default	cure	not-available	6	7
26	35/M	default	default	–	14	–
27	52/M	failure	cure	–	7	–
28	43/F	failure	failure	cure	6	8
29	61/F	failure	failure	–	5	–
30	62/M	failure	cure	–	10	–
31	51/M	failure	cure	–	6	–
32	44/F	failure	complete	–	5	–
33	47/M	failure	failure	died	4	10
34	30/M	failure	cure	–	5	–
35	33/F	failure	cure	–	14	–
36	65/M	failure	failure	failure	3	3
37	53/F	failure	cure	–	6	–
38	42/M	failure	cure	–	13	–
39	65/M	failure	default	–	5	–
40	43/M	failure	default	–	8	–
41	58/M	failure	default	–	18	–
42	59/M	failure	cure	–	7	–

Resistance to anti-tuberculosis drugs

Table 2 summarizes anti-TB drug resistance among *M. tuberculosis* isolates from recurrent TB patients in relation to treatment outcomes. In the first, second and third registrations, monodrug resistant strains from successful and unsuccessful TB treatment comprised 7.3% (3/41), 5.0% (2/40) and 12.5% (1/8), respectively. Polydrug resistant strains after unsuccessful TB treatment comprised 4.9% (2/41), 2.5% (1/40) and 25.0% (2/8), respectively. MDR strains after unsuccessful TB treatment comprised 12.2% (5/41), 20.0% (8/40) and 12.5% (1/8), respectively. We found one MDR-TB strain in a case of successful TB treatment (2.5%). In patients with retreatment TB after unsuccessful treatment (failure and default), MDR ($P = 0.025$) and drug resistance to isoniazid ($P = 0.025$) and to rifampin ($P = 0.046$) occurred significantly less frequently in first registration than in second registration *M. tuberculosis* isolates. However, we found no significant differences in resistance to any TB drugs in isolates from the first and second registrations of patients with relapsed TB after successful treatment (completed and cure). We found no significant differences between first and second registrations in resistance of *M. tuberculosis* to ethambutol ($P = 0.157$) and to streptomycin ($P = 0.564$) in patients with retreatment after unsuccessful treatment (failure and default). Obviously, we more commonly found acquired MDR-TB strains in patients with failure (6/16) and default treatment (2/4), and rarely in those with successful treatment (1/22). Interestingly, two acquired MDR-TB strains from patients with retreatment after treatment failure developed resistance to all anti-TB drugs tested including RMP, INH, SM and EMB.

Insertion sequence *6110*-restriction fragment length polymorphism patterns

The IS*6110*-RFLP patterns of *M. tuberculosis* isolates can be classified into five groups as previously described (20). Figure 1 shows sampling examples of patients with identical IS*6110*-RFLP patterns and two patients with

Table 2. Development of anti-TB drug resistance among *M. tuberculosis* isolates from recurrent TB patients in relation to treatment outcomes

DST results	Resistant isolates		
	First registration Number (%)	Second registration Number (%)	Third registration Number (%)
Successful treatment			
Monodrug resistance	3 (7.3)	2 (5.0)	1 (12.5)
Rifampin	0	0	0
Isoniazid	2 (4.9)	1 (2.5)	1 (12.5)
Streptomycin	0	0	0
Ethambutol	1 (2.4)	1 (2.5)	0
Polydrug resistance	0	0	0
MDR	0	1 (2.5)	0
Unsuccessful treatment			
Monodrug resistance	3 (7.3)	2 (5.0)	1 (12.5)
Rifampin	0	0	0
Isoniazid	1 (2.4)	1 (2.5)	0
Streptomycin	0	0	0
Ethambutol	2 (4.9)	1 (2.5)	1 (12.5)
Polydrug resistance	2 (4.9)	1 (2.5)	2 (25.0)
MDR	5 (12.2)	8 (20.0)	1 (12.5)

DST, drug susceptibility testing.

Table 3. IS*6110*-RFLP patterns of *M. tuberculosis* in recurrent TB patients in the first, the second and the third registrations

IS*6110*-RFLP patterns	Number of patients		
	First registration	Second registration	Third registration
Identical patterns			
Single band	5	5	1
2–5 bands	5	5	0
Beijing	21	21	6
Nonthaburi	3	3	1
Heterogeneous	6	6	0
Non- identical patterns			
2–5 bands	1	1	0
Beijing	1	1	0

non-identical patterns and Table 3 shows the numbers of recurrent TB patients with various IS*6110*-RFLP patterns. We found identical patterns in 40/42 isolates (95%) from recurrent patients and non-identical patterns in 2 isolates (5%) in isolates from patients with retreatment after failure. Among 40 isolates with identical patterns, 21 (50.0%) belonged to the Beijing family with 15–20 copies and 3 (7.1%) to the Nonthaburi family with 11–15 copies patterns. We were unable to group other isolates from six patients (14.3%) with heterogeneous patterns with more than five bands as either Beijing or Nonthaburi families. Five patients (11.9%) had isolates hybridized at only one position and were either 1.45 kb or 1.3 kb long; these isolates were likely to contain only a single copy of IS*6110* (defined as single band pattern). We defined the isolates

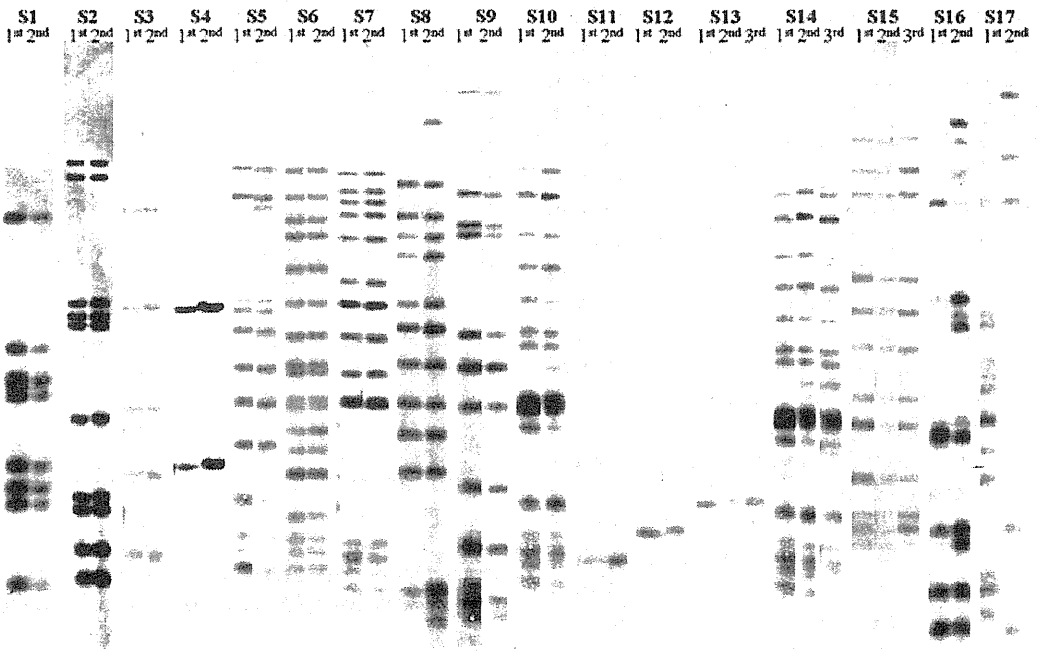

Fig. 1. Identical and non-identical IS*6110*-RFLP patterns of *M. tuberculosis* isolates from seventeen pulmonary TB patients. Identical patterns: S1–S12 are subjects 1–12 from two registrations and S13–S15 are subjects 13–15 from three registrations. Non-identical patterns: S16–S17 are subjects 16 and 17 from two registrations.