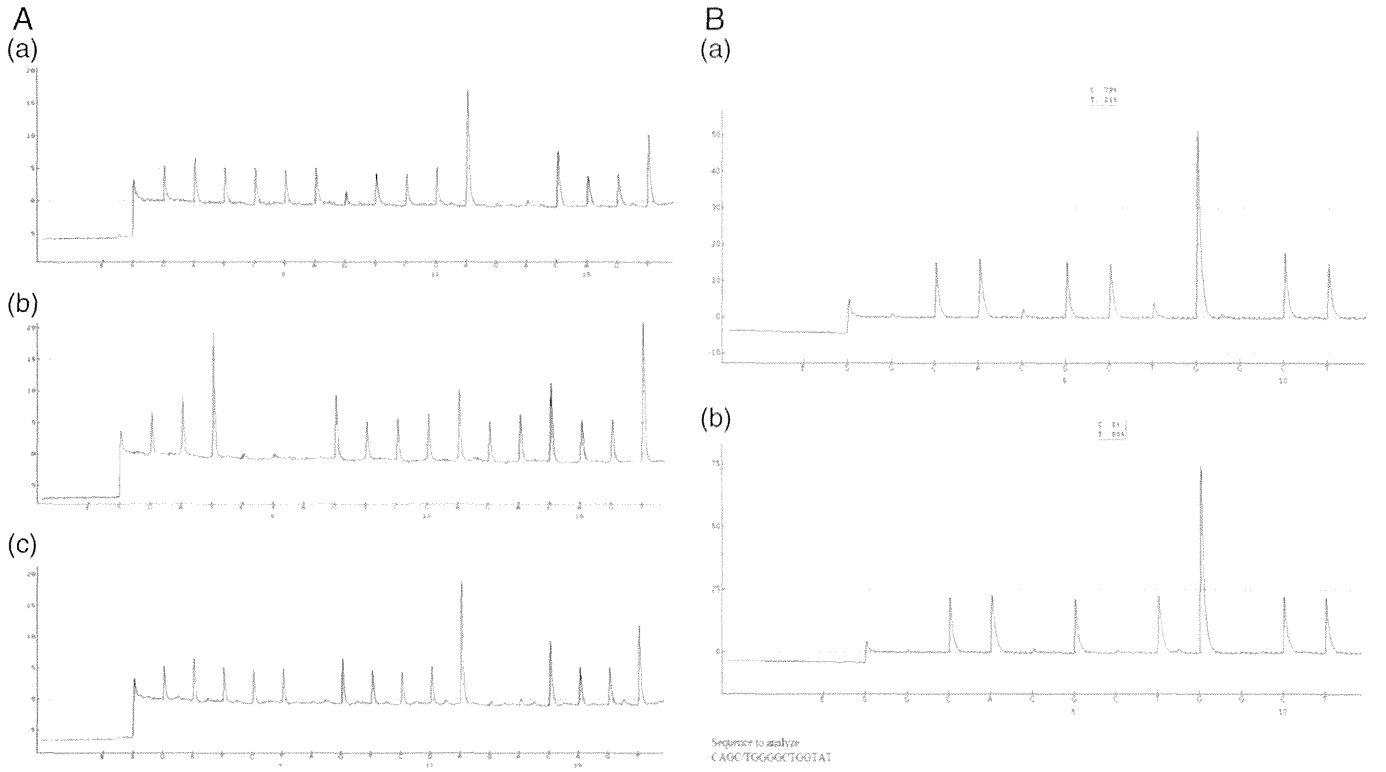

Fig. 2. Representative pyrosequencing of the *erm*(41) gene with the F1R1 primer set. The pyrograms in (A) are for identification of (a) *M. abscessus*, (b) *M. massiliense*, and (c) *M. bolletii*, and the pyrograms in (B) are for sequevars of (a) C28 and (b) T28.

4. Discussion

We designed pyrosequencing assays for the *erm*(41) gene because this reliable, fast, and labor-saving sequencing technology will facilitate rapid molecular detection of clarithromycin resistance in clinical isolates. We also developed a pyrosequencing method for detecting mutations at positions 2058 and 2059 of the 23S rRNA gene, in accordance with a previous report (Haanpera et al., 2005). This is important because, in the *M. abscessus* group, resistance to macrolides is generally acquired through mutation of the *rrl* gene that encodes the 23S rRNA (Wallace et al., 1996). In this study of strains from the *M. abscessus* group, the identities determined by pyrosequencing were completely consistent with those determined by multi-gene sequencing and *erm*(41) genotyping (Bastian et al., 2011). However, long-read multi-gene sequencing is subject to practical limitations in terms of assays and the complexities of testing additional sites. In our experience, it is difficult for medical laboratory technicians to meet the turnaround times required for rapid microbiological identification using traditional multi-gene sequencing. Although the results of standard *erm*(41) gene sequencing were correct, *erm*(41) genotyping by pyrosequencing was much faster. Conventional methods of phenotypic identification are also time consuming. In contrast, we observed that pyrosequencing of the *erm*(41) gene provided rapid, robust, and reliable identification. Unlike conventional sequencing, no purification of PCR products was required for the assay. Therefore, we believe pyrosequencing is a simple and reliable tool for identifying *M. abscessus* group isolates.

The *M. massiliense erm*(41) gene includes several mutations, including a large C-terminal deletion that renders it nonfunctional. Analysis of the size differences of the products obtained with *erm*(41) PCR was proposed as a simple method to differentiate *M. massiliense* from *M. abscessus* and *M. bolletii* species (Kim et al., 2010). Shallom et al. (2013) combined this scheme with *erm*(41) PCR and identified 2 *M. massiliense* isolates that harbored full-length *erm*(41) PCR products. Shallom and coauthors concluded that the *erm*(41) PCR method

Table 2
In vitro clarithromycin susceptibility and inducible clarithromycin resistance in the *M. abscessus* group.

Isolates	n	Read on day	No. strains per MIC (mg/L)													Range	MIC_{50}	MIC_{90}	Susc.	Int.	Res.	*P* values
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64							
M. abscessus	28																					
T28 sequevar	25	3				1	3	5	5	2	7		**1 [0]**[a]		**1 [0]**[a]	0.12–64	1	4	16 (64)	7 (28)	2 (8)	0.0009[b]
		14								1		**1**	**3**	**17**	**3**							
C28 sequevar	3	3				3										0.12	-	-	3 (100)	0 (0)	0 (0)	
		14				2	1															
M. massiliense	25	3		1	4	11	4	4	1							0.03–1	0.12	0.5	25 (100)	0 (0)	0 (0)	
		14		1	4	8	7	4	1													
M. bolletii	2	3													**2 [1]**[a]	64	64	64	0 (0)	0 (0)	2 (100)	
		14													**2**							

Susc = susceptible; Int = intermediate; Res = resistant.
[a] Number of highly resistant strains with the A2058G or A2059G SNP in the 23S rRNA gene.
[b] Significant difference between the susceptibilities of *M. abscessus* T28 sequevar and *M. massiliense* was revealed by a χ^2 test.

described by Kim et al. (2010) is insufficient to differentiate *M. massiliense* from *M. abscessus*. Based on the small set of strains in this study, we did not find an association between *M. massiliense* with the full-length *erm*(41) product and inducible clarithromycin resistance, but this association may be observed in additional studies of larger, more diverse samples. Several authors have suggested that single-target sequencing may lead to inaccurate identification (Leao et al., 2011; Nakanaga et al., 2011; Zelazny et al., 2009). Our pyrogram patterns were unambiguous in the majority of the clinical isolates tested. However, *M. abscessus* group identification using single-target pyrosequencing may not be adequate to resolve the breadth of species diversity by sequence variability within the short targeted stretch of *erm*(41). A more-diverse selection of strains should be tested to verify the discriminatory power of the assay for differentiating the 3 species.

Our method is designed to simultaneously provide identification and *erm*(41) genotyping, which are easy to perform even without gel dye. Our pyrosequencing method offers rapid identification of *M. abscessus*, *M. massiliense*, and *M. bolletii* by sequencing the upstream region of *erm*(41) and detection of nucleotide substitutions associated with inducible clarithromycin resistance. Our protocol does not require an expensive platform and is a relatively simple technique that provides visual results in real time.

M. abscessus infections tend to respond poorly to macrolide-based chemotherapy because of inducible and acquired resistance (Bastian et al., 2011; Harada et al., 2012; Koh et al., 2011). The *erm*(41) gene confers inducible resistance to clarithromycin in clinical isolates of *M. abscessus* (Bastian et al., 2011; Nash et al., 2009), and resistance to macrolides can be acquired through mutations in the *rrl* gene encoding 23S rRNA (Wallace et al., 1996). Wallace et al. (1996) sequenced the gene encoding the 23S rRNA region, revealing the presence of a point mutation involving adenine at position 2058 (38%) or 2059 (62%). They reported that this particular point mutation strongly correlates with phenotypic resistance.

We sought to predict inducible resistance based on 2 PCR primer sets (F1R1 and F3R3) that were used for identification, thereby confirming that the sequence of interest was indeed 2 sequevars (T28 and C28). Notably, we found that the 14-day MICs for clarithromycin were lower for isolates of *M. massiliense* than for the *M. abscessus* and *M. bolletii* (Table 2), suggesting greater susceptibility of *M. massiliense* to clarithromycin. The sequevars that we identified by pyrosequencing the *erm*(41) gene were highly predictive of resistance induced by microdilution. Pyrosequencing has also been used to detect and quantify macrolide resistance mutations at positions 2058 and 2059 of the 23S rRNA gene for *Mycobacterium avium* (Haanpera et al., 2005). In this study, however, 3 isolates with acquired resistance did not have mutations at either of these positions. As presented in Table 2, our pyrosequencing array did not detect high mutation frequencies among isolates with acquired resistance (25.0% for *rrl* in acquired-resistant isolates). The predominance of mutations in the *rrl* gene encoding 23S rRNA differs from previous findings (Nessar et al., 2012; Wallace et al., 1996); this predominance has been described as characteristic of Korean and Japanese strains (Kim et al., 2008, 2010; Yoshida et al., 2013). Indeed, we believe this polymorphism could be a marker of geographic origin (Yoshida et al., 2013). On the other hand, alternative genotypic methods of determining acquired clarithromycin resistance are limited because the mechanisms of acquired resistance in the *M. abscessus* group remain unknown (Kim et al., 2008, 2010; Yoshida et al., 2013). Consequently, pyrosequencing cannot replace conventional phenotypic susceptibility analysis.

Previous reports have determined that clarithromycin-based antibiotic therapy for *M. massiliense* lung disease is associated with higher response rates than similar therapy for *M. abscessus* lung disease (Harada et al., 2012; Koh et al., 2011). Therefore, prior assessment of clarithromycin susceptibility is necessary to provide appropriate treatment for lung disease. In this study, pyrosequencing of the *erm*(41) gene successfully identified the 3 species of the *M. abscessus* group. Moreover, our assay can identify sequevars (T28 or C28) that can be used to assess clarithromycin activation for patients undergoing treatment. Compared with conventional testing, our methods provided reliable results for inducible clarithromycin susceptibility and can provide an excellent marker for detecting inducible resistance to the most important clarithromycin medications.

In conclusion, pyrosequencing presents several key advantages. It is faster than conventional methods for determining susceptibility and can improve turnaround times. It also facilitates large-volume testing and costs less than molecular tests that require multiple probes. Pyrosequencing of the *erm*(41) gene exhibits excellent agreement with standard multiple gene sequencing and *erm*(41) sequencing (Bastian et al., 2011). To our knowledge, this is the first study to provide a pyrosequencing method for monitoring regions in the *erm*(41) gene to detect sequevars (based on the T/C polymorphism at nucleotide 28) that are associated with clarithromycin-inducible resistance. Pyrosequencing data can provide useful information for physicians. The rapid availability of sequevar data will allow appropriate treatment to be established substantially faster than would be possible using conventional testing. The limited number of strains, particularly *M. bolletii*, in this study suggests it is too early to judge if all strains in this group can be fully differentiated by a single-target sequencing assay. Further studies analyzing more diverse strains from different locations should be tested to verify our conclusions.

Acknowledgments

Ryoko Shimada is an employee of QIAGEN K.K. (Tokyo, Japan). There are no other competing interests to declare.

We have no relevant affiliations or financial involvement relating to the subject matter or materials discussed. The study was not supported by external agencies.

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Journal of Medical Microbiology (2015), 64, 254–261 DOI 10.1099/jmm.0.000016

Investigation of the population structure of *Mycobacterium abscessus* complex strains using 17-locus variable number tandem repeat typing and the further distinction of *Mycobacterium massiliense hsp65* genotypes

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Mycobacterium abscessus complex is a significant pathogen in patients with non-cystic fibrosis (non-CF). Nevertheless, there is little description of the genetic diversity of this species. The aims of this study were to investigate the distribution of *M. abscessus* complex isolated from respiratory specimens by variable number tandem repeat (VNTR) typing. The results of 104 clinical isolates from 104 non-CF patients were compared using PFGE, *hsp65* genotypes and clarithromycin susceptibility. The allelic diversity (Hunter–Gaston Discriminatory Index) of the 17 loci examined by VNTR typing was high (0.977). We determined that C28 sequevar *erm*(41) genotypes and clarithromycin-acquired resistance isolates were scattered in the minimum spanning tree. Intriguingly, VNTR typing and PFGE were highly congruent and revealed that there were clear examples of grouping of isolates from different individuals amongst both *M. abscessus* and *M. massiliense*, and showed five clusters of distinct identical isolates. Within these clusters, *M. massiliense hsp65* type I formed three different clusters. Although the distribution of *M. massiliense hsp65* type II-1 was low (9.3%), *M. massiliense hsp65* type II-1 isolates separated from clusters contained *hsp65* type I isolates. Thus, *M. massiliense hsp65* genotypes could be discriminated by analysing VNTRs with sufficient genetic distance for intra-species-level discrimination.

Received 23 October 2014
Accepted 23 December 2014

INTRODUCTION

The *Mycobacterium abscessus* complex (*M. abscessus sensu lato*) contains rapidly growing mycobacteria that have been increasingly recognized as opportunistic human pathogens (Choi *et al.*, 2011). The *M. abscessus* complex comprises ubiquitous environmental micro-organisms that are frequently associated with hospital-acquired outbreaks and pseudo-outbreaks (Brown-Elliott & Wallace, 2002; Duarte *et al.*, 2009; Fisher & Gloster, 2005; Monego *et al.*, 2011; Nunes *et al.*, 2014). The subspecies of the *M. abscessus* complex have undergone taxonomical nomenclature changes,

Abbreviations: CF, cystic fibrosis; HGDI, Hunter–Gaston Discriminatory Index; MICs, minimum inhibitory concentrations; MLST, multi-locus sequence typing; MST, minimum spanning tree; non-CF, non-cystic fibrosis; PRA, PCR restriction analysis; VNTR, variable number tandem repeat.

previously classified as three separate subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (Adékambi & Drancourt, 2004; Adékambi *et al.*, 2006). The currently accepted nomenclature places *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* into a single subspecies called *M. abscessus* subsp. *bolletii* (Leao *et al.*, 2011). However, whole-genome sequencing supported the separation as three subspecies (Bryant *et al.*, 2013). We concur that there has been some debate in the literature regarding the taxonomy of these organisms (Harris & Kenna, 2014). In this report, we describe *M. abscessus* complex isolates as three species [*M. abscessus sensu stricto* (*M. abscessus*), *M. massiliense* and *M. bolletii*] to enable the comparison of macrolide-acquired and inducible resistance between *M. abscessus* and *M. massiliense*. Inducible resistance is conferred by *erm*(41) – one of the few antibiotic resistance genes of the *M. abscessus* complex to be characterized, the truncated sequence seen in *M. massiliense* or the point mutation (C28 sequevar) seen in some *M. abscessus* strains, both of which result in a non-functional *erm*(41) gene (Bastian *et al.*, 2011; Harris & Kenna, 2014).

Epidemic strain typing has identified dominant strains of *M. massiliense* that have caused widespread soft-tissue infections following surgery (Cheng *et al.*, 2013; Duarte *et al.*, 2009). Intriguingly, a new genomic study revealed an unexpectedly high genetic similarity between the dominant isolates in Brazil and respiratory isolates from an outbreak of *M. massiliense* in cystic fibrosis (CF) patients in the UK (Davidson *et al.*, 2013). Moreover, the Brazilian epidemic isolates, the UK outbreak isolates and the M18 strain from non-cystic fibrosis (non-CF) Malaysian patients belonged to a monophyletic clade. By contrast, other Malaysian strains (M148, M156 and M172; Wong *et al.*, 2012) isolated over the same time frame as the Brazilian and UK outbreak strains were genetically diverse and mostly distinct from these strains (Davidson *et al.*, 2013).

The frequency of *M. massiliense* isolation appears to vary regionally (Harada *et al.*, 2012; Kim *et al.*, 2008; van Ingen *et al.*, 2009; Zelazny *et al.*, 2009). Recently, it was further reported that *M. massiliense* in Korean patients could be subdivided into two genotypes based on *hsp65* sequence analysis (Kim *et al.*, 2012). In their study, Kim *et al.* (2012) found that all *M. massiliense* strains belonging to the *hsp65* genotype II, which have a rough colony morphotype without any exceptions, differed from other isolates of *M. abscessus* complex showing either smooth or rough colony morphotypes. Moreover, the clinical outcome of patients with *M. massiliense hsp65* type II after antimicrobial treatment in previous *M. massiliense* infection might be better than in *M. abscessus* infection (Jeon *et al.*, 2014). However, there have not been any reports on the distribution of *M. massiliense hsp65* type II from areas located outside of Korea.

To better define particular lineages more frequently associated with human infection, polyphasic genotyping studies are increasingly being employed. Variable number tandem repeat (VNTR) typing provides a sophisticated method of analysing genetic polymorphism. In this study, we aimed to provide insights into the population structure of clinical *M. abscessus* complex isolates by VNTR typing. To define the genetic relatedness amongst the geographical distant isolates, we compared VNTR profiles between isolates in Japan and those previously reported for Malaysian isolates including strains (M148, M156, M172 and M18) (Wong *et al.*, 2012). We also investigated the genetic distribution of *hsp65* genotypes in *M. massiliense* isolates. To analyse the association between clustered isolates and clarithromycin susceptibility with *erm*(41) sequevars, susceptibility testing and *erm*(41) genotyping of these isolates were performed.

To our knowledge, this is the first report from Japan analysing VNTR profiles and the distribution of *hsp65* genotypes of *M. massiliense*. None has clinical features of CF in our study because the incidence of CF is normally rare in Asians and there is very little information available from most Asian countries, including Japan (WHO, 2004).

METHODS

Bacterial isolates. Previously, from 104 clinical isolates, three species of *M. abscessus* complex were identified by sequencing the 16S rRNA, *hsp65* and *rpoB* genes (Kim *et al.*, 2012; Yoshida *et al.*, 2013). The identities determined using these sequencing assays agreed completely; on the basis of the results, we subdivided the 104 clinical isolates into 59 *M. abscessus*, 43 *M. massiliense* and two *M. bolletii* isolates, and the reference strains (*M. abscessus* JCM13569T, *M. massiliense* JCM15300T and *M. bolletii* JCM15297T). The 104 isolates were collected from Osaka (western Japan), Tokyo (central Japan) and Hokkaido (northern Japan), which are 600–800 km apart from each other, and were obtained from the sputa of patients with pulmonary *M. abscessus* complex infection. These patients were treated at the National Hospital Organization Kinki-chuo Chest Medical Center in western Japan (56 isolates from 56 patients; 28 *M. abscessus*, 26 *M. massiliense* and two *M. bolletii*) between 2008 and 2010; at the Japan Community Health Care Organization Hokkaido Hospital in northern Japan (14 isolates from 14 patients; eight *M. abscessus* and six *M. massiliense*) between 2000 and 2013; and at two hospitals in central Japan: National Hospital Organization Tokyo National Hospital (14 isolates from 14 patients; 12 *M. abscessus* and two *M. massiliense*) between 2007 and 2009, and Fukujuji Hospital (20 isolates from 20 patients; 13 *M. abscessus* and seven *M. massiliense*) between 2005 and 2009. All patient samples tested negative for human immunodeficiency virus. The Ethics Committee of National Hospital Organization Kinki-chuo Chest Medical Center approved this study.

***erm*(41) and *hsp65* genotyping.** To extract DNA, a loopful of colonies of each strain was used, and the extracted DNA was suspended in 300 µl 1× TE (Tris-EDTA) buffer and boiled for 10 min. For PCR amplifications, crude lysates were used. The *erm*(41) gene region was amplified using the primers *erm*F (5′-GACCGGG-GCCTTCTTCGTGAT-3′) and *erm*R1 (5′-GACTTCCCCGCACCGA-TTCC-3′) (Kim *et al.*, 2010). The PCR cycling conditions were: initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 10 min. Mutations in the 23S rRNA gene (*rrl*) were detected by PCR sequencing as described

previously (Bastian *et al.*, 2011). The *M. massiliense* isolates were further classified at the subspecies level based on the results of *hsp65* sequencing and the *Hin*fI PCR restriction analysis (PRA) method (Kim *et al.*, 2012).

Clarithromycin susceptibility testing. Clarithromycin MICs were determined in cation-adjusted Mueller–Hinton medium by using the broth microdilution method. Plates were evaluated after 3 days and then incubated for 14 days at 35 °C in order to obtain a final reading to ensure the detection of inducible resistance. The interpretative breakpoints used were those recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

VNTR typing and minimum spanning tree (MST) generation. The epidemiological data comprised mycobacterial DNA interspersed with repetitive units of VNTRs targeting the 18 loci (Wong *et al.*, 2012). However, we excluded the TR2 locus from our analysis because no stable size difference between *M. abscessus* and *M. massiliense* isolates was measured. The allelic diversity of each VNTR locus was evaluated and the level of genotypic diversity of each VNTR locus set was calculated using the Hunter–Gaston Discriminatory Index (HGDI) (Hunter & Gaston, 1988). For use in clustering analysis, a MST was generated on the basis of the 17-locus VNTR profiles, in which our own data were combined with the Malaysian data (Wong *et al.*, 2012) using BioNumerics 4.6 software (Applied Maths). To generate the MSTs, we used the reconstruction rules described elsewhere (Iwamoto *et al.*, 2012).

PFGE. All of the large clustered isolates (48 isolates) of the VNTR analysis and the reference strains of *M. abscessus* and *M. massiliense* were analysed genotypically using PFGE. As the numbers of *M. bolletii* were small and unclustered by the VNTR analysis, these strains were not included in PFGE. Thiourea (100 μmol l^{-1}) was added to the gel running buffer. All the parameters of the PFGE procedures, including DNA preparation and restriction endonuclease digestion with *Dra*I (Roche Diagnostics), were the same as those set previously (Duarte *et al.*, 2009), except for the pulse times. Restriction fragments were separated using agarose gels (1 %) and a CHEF-DRIII electrophoresis system (Bio-Rad), and the pulse times were increased from 1.4 to 21.3 s over 19 h at 14 °C, at a voltage gradient of 6 V cm^{-1} and an included angle of 120°. The PFGE patterns were analysed using commercial molecular fingerprinting software Phoretix and Phoretix 1D (Nonlinear Dynamics). This comparison was performed using the Ward algorithm and calculated using the Jaccard correlation coefficient.

RESULTS

The *erm*(41) genotyping was completely consistent with the published *erm*(41) gene sequences (Kim *et al.*, 2010). We determined that six *M. abscessus* isolates featured a C28 sequevar in *erm*(41) and the remaining 53 were T28 sequevar isolates. The 43 *M. massiliense* isolates were further classified into three genotypes based on 644 bp *hsp65* sequences and the results obtained using the *Hin*fI PRA method, as previously described (Kim *et al.*, 2012). In total, 39 clinical isolates were classified as *hsp65* type I and four isolates were classified as *hsp65* type II-1; however, no isolates were classified as type *hsp65* II-2.

We determined that 99/104 isolates were susceptible to clarithromycin at day 3 ($\leqslant$8 μg ml^{-1}) and that there were five clarithromycin-resistant isolates (>32 μg ml^{-1}). These resistant isolates contained four *M. abscessus* isolates (T28 sequevar) and one *M. bolletii* isolate; only one *M. abscessus* isolate did not have a point mutation in *rrl*. On day 14, 49 *M. abscessus* isolates (T28 sequevar) and one *M. bolletii* isolate were even more resistant to clarithromycin than on day 3. These isolates developed inducible resistance to clarithromycin *in vitro*, but six *M. abscessus* isolates (C28 sequevar) showed stable clarithromycin susceptibility. By contrast, clarithromycin MICs of all 43 *M. massiliense* isolates showed susceptibility on day 3 and remained at this level during the 14-day observation period (these isolates were considered susceptible).

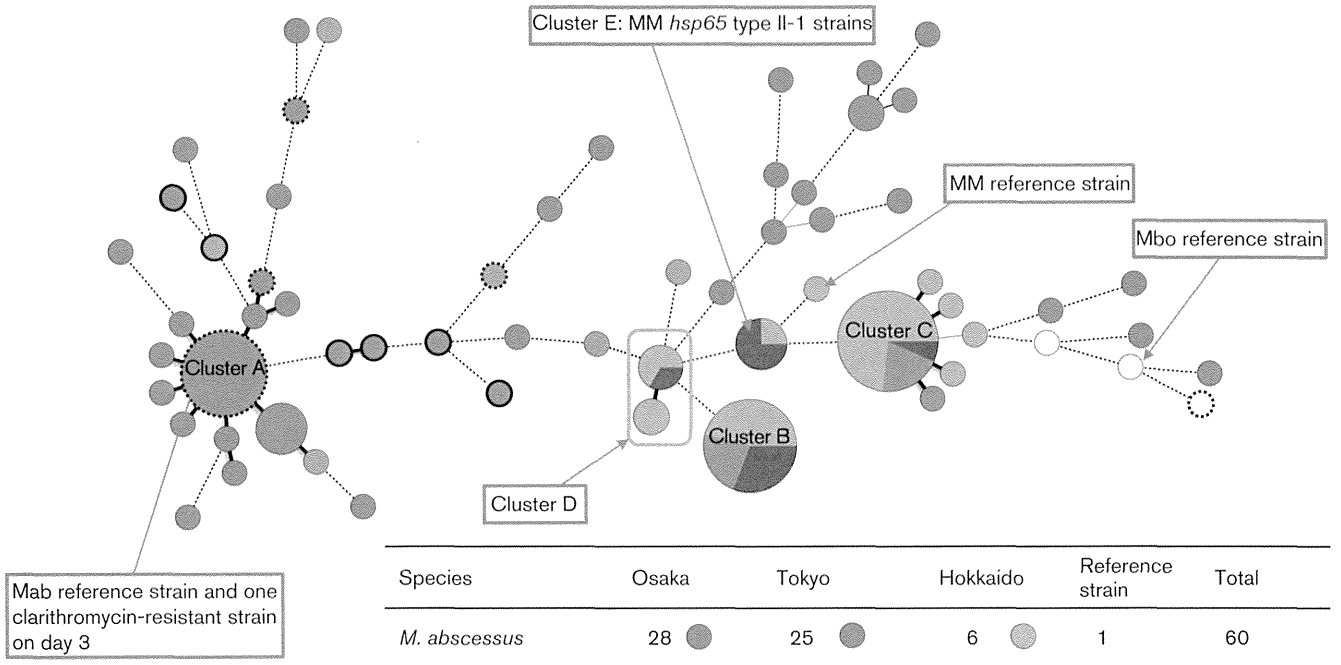
The 17-locus VNTR analysis grouped the 104 isolates into 62 profiles. The discriminatory power (HGDI) using the 17-locus VNTR analysis reached a peak of 0.977. The diversity levels in the 17 loci were increased compared with that determined using only six loci (HGDI=0.9417; TR150, TR172, TR155, TR109, TR116 and TR45).

Using the MST, we clearly distinguished *M. massiliense* and *M. abscessus* isolates (Fig. 1). However, *M. bolletii* isolates and the reference strain (JCM15297T) were not separated from the *M. abscessus* and *M. massiliense* isolates. The VNTR profiles of the isolates were combined on the basis of similar and distinct patterns according to their geographical origin (Osaka, Tokyo or Hokkaido). The *M. abscessus* Cluster A isolates exhibited the same profile as that of the reference, *M. abscessus* JCM13569T. We identified further four clusters in *M. massiliense* (Clusters B–E); these clusters comprised 13, 15, five (including three isolates with five copies and two isolates with four copies in TR109) and four isolates, respectively.

None of the C28 sequevar isolates shared identical VNTR profiles with either the *M. abscessus* large cluster (Cluster A) or the remaining T28 sequevar isolates (Fig. 1). Although one acquired resistance isolate formed in Cluster A, we determined that the remaining four clarithromycin-acquired resistance isolates were scattered in the MST (Figs 1 and 2). Although Clusters B–D comprised *M. massiliense hsp65* type I isolates depending on the *hsp65* genotype, Cluster E comprised *M. massiliense hsp65* type II-1 isolates.

We compared our 17-locus VNTR data with the data obtained from the Malaysian isolates by creating a MST (Fig. 2). The clustered profiles of Malaysian isolates clearly matched one consistent cluster of *M. abscessus* (Cluster A) and two consistent clusters of *M. massiliense* (Clusters B and C) in the present study. The profile of 10 Cluster A isolates from Japan was the same as that of M61 and M68 from Malaysia. Similarly, the profile of 13 Cluster B isolates was the same as that of six Malaysian isolates (M18, M2, M4, M27, M145 and M163) and the profile of 15 Cluster C isolates was the same as that of three Malaysian isolates (M148, M156 and M172). However, no Malaysian isolates matched the profiles of Clusters D and E exactly.

The 48 isolates tested were grouped into 47 PFGE patterns (Fig. 3). The PFGE patterns were divided into five clusters and the PFGE scheme mostly grouped isolates in



Species	Osaka	Tokyo	Hokkaido	Reference strain	Total
M. abscessus	28	25	6	1	60
M. massiliense	26	9	8	1	44
M. bolletii	2	0	0	1	3

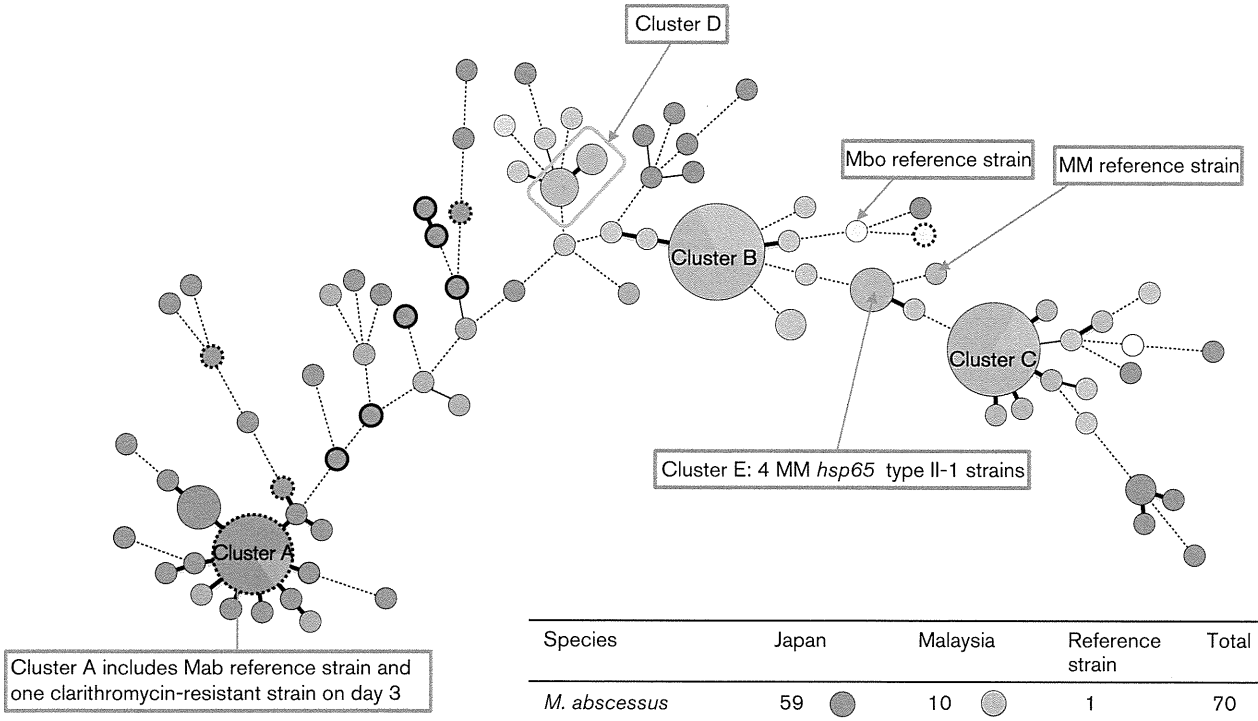
Fig. 1. An MST constructed on the basis of the 17-locus VNTR profiles of *M. abscessus* complex isolates (104 isolates from Japan: 56 isolates from Osaka, 34 isolates from Tokyo and 14 isolates from Hokkaido) and reference strains. Circle sizes are proportional to the numbers of isolates sharing an identical pattern. *M. abscessus* strains were isolated from Osaka (red), Tokyo (purple) and Hokkaido (pink); *M. massiliense* strains were isolated from Osaka (sky blue), Tokyo (blue) and Hokkaido (green); *M. bolletii* strains were isolated from Osaka only (yellow). Heavy-lined circles indicate clarithromycin-resistant strains on day 3, as determined on the basis of susceptibility testing. Dot-lined circles indicate clarithromycin-susceptible strains on day 14 and the C28 genotype of the *erm*(41) gene. Heavy lines connecting two types denote single-locus variants, thin lines connect double-locus variants and dotted lines connect triple-locus variants or indicate the most likely connection between two types differing by more than three VNTR loci. Mab, *M. abscessus*; MM, *M. massiliense*; Mbo, *M. bolletii*.

agreement with VNTR. However, PFGE showed that the reference strain JCM15300T was not differentiated from the Cluster B isolates of VNTR. These clustered strains had been isolated from three different locations between 2000 and 2013.

DISCUSSION

PFGE is used extensively to analyse epidemiological strains within the *M. abscessus* complex and monitor outbreaks (Cheng *et al.*, 2013; Zelazny *et al.*, 2009). In a previous report, *M. massiliense hsp65* type II isolates were sub-divided clearly by two molecular typing methods: PFGE and multi-locus sequence typing (MLST) (Jeon *et al.*, 2014). However, PFGE has been used to type species to the strain level using analogue band patterns; therefore, it is difficult to compare large results from distinct experiments and laboratories. The MLST scheme showed a valuable type ability for *M. abscessus* complex (Macheras *et al.*, 2014), and could be used by clinical laboratories to assess relatedness of newly isolated strains to the global cluster (Tettelin *et al.*, 2014). However, the MLST was less discriminative than PFGE for *M. abscessus*(Machado *et al.*, 2014) and the VNTR performed better than the MLST in the strain differentiation of *M. abscessus*(Wong *et al.*, 2012). However, MLST can be used by clinical laboratories to assess the relatedness of newly isolated strains to the global cluster (Tettelin *et al.*, 2014).

Notably, using VNTR for digital epidemiological methods in this study could yield insights into the genetic distribution of *M. abscessus* and *M. massiliense*. The relatedness of the diverse *M. abscessus* complex was clear between Japanese and Malaysian isolates, including non-outbreak isolates (M148, M156, M172 and M18). However, it remained unclear why organisms from both countries were so closely related. One hypothesis is that there are common infectious sources at the global level, such as environmental opportunistic pathogens of humans and animals, which are transmitted



Species	Japan	Malaysia	Reference strain	Total
M. abscessus	59	10	1	70
M. massiliense	43	24	1	68
M. bolletii	2	1	1	4

Fig. 2. An MST constructed on the basis of the 17-locus VNTR profiles of 139 *M. abscessus* complex isolates (104 isolates from Japan and 35 isolates from Malaysia) and reference strains. Circles correspond to the distinct types discriminated on the basis of 17 VNTR genotypes and circle sizes are proportional to the numbers of isolates sharing an identical pattern. *M. abscessus* strains were isolated from Japan (red) and Malaysia (pink); *M. massiliense* strains were isolated from Japan (blue) and Malaysia (sky blue); *M. bolletii* strains were isolated from Japan (yellow) and Malaysia (orange). Heavy-lined circles indicate clarithromycin-resistant strains on day 3, as determined on the basis of susceptibility testing. Dot-lined circles indicate clarithromycin-susceptible strains on day 14 and the C28 genotype of the *erm*(41) gene. Heavy lines connecting two types denote single-locus variants, thin lines connect double-locus variants and dotted lines connect triple-locus variants or indicate the most likely connection between two types differing by more than three VNTR loci. Mab, *M. abscessus*; MM, *M. massiliense*; Mbo, *M. bolletii*.

between the environment, wildlife and imported/exported livestock. *M. abscessus* strains from potable water were indistinguishable from those causing infection in humans from the same geographical area (Thomson *et al.*, 2013). Another possibility is that strains with potentially higher transmissibility have been distributed globally through humans. According to previous molecular investigations in CF patient groups, *M. abscessus* complex isolates from the majority of individual patients were indistinguishable by both nine-locus VNTR analysis and the Diversilab rep-PCR typing method; moreover, the same strain or genotype was found with no apparent epidemiological links (Harris *et al.*, 2012). Whole-genome sequencing demonstrated that the clustering strains might be more transmissible in CF patient groups (Bryant *et al.*, 2013). According to reports of global epidemiological studies, MLST is useful for the global comparison of strains; the most prevalent sequence types were ST1 (CC5; *M. abscessus* isolates) and ST23 (CC3; *M. massiliense* isolates), and these sequence types were found from Europe and Brazil in different years (Macheras *et al.*, 2014). Intriguingly, ST23 isolates were demonstrated in a large post-surgical procedure outbreak in Brazil (Duarte *et al.*, 2009; Macheras *et al.*, 2014). Although no information on MLST sequence types was available for our isolates, our VNTR clusters were grouped into large clonal complexes as well as global epidemiological clones that were consistent with MLST (Macheras *et al.*, 2014). Furthermore, Malaysian M172 strain, one of the Cluster C strains in the VNTR analysis, was grouped to the Cluster 2B (C2B) strains isolated from Nepalese and English patients by whole-genome sequencing (Sassi & Drancourt, 2014). In contrast, M18 with the Cluster B VNTR profile belongs to the C2A

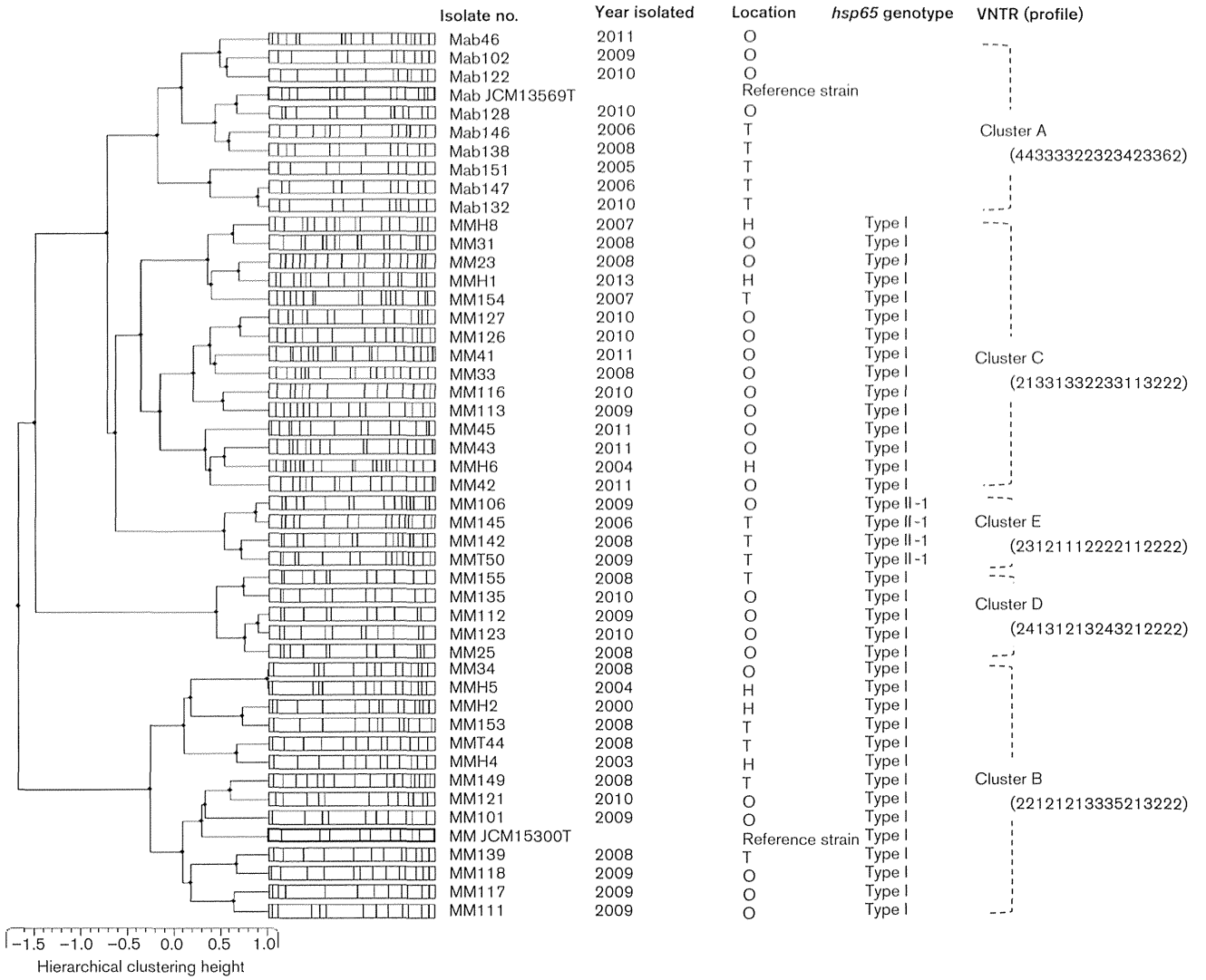


Fig. 3. Comparison between the PFGE dendrogram produced using *Dra*I and clusters based on VNTR profiles of *M. abscessus* and *M. massiliense*. Species assignment based on 16S rRNA gene, *hsp65* and partial *rpoB* sequence; Mab, *M. abscessus*; MM, *M. massiliense*. Location: O, Osaka; T, Tokyo; H, Hokkaido. VNTR clusters correspond to those in the MSTs in Figs 1 and 2.

strains from other Malaysian and French patients (Sassi & Drancourt, 2014). Our study did not consider epidemiological background or transmission routes amongst *M. abscessus* complex infectious patients. No epidemiological data (incidence rate and prevalence) on respiratory infection by *M. abscessus* complex were collected during a nationwide surveillance program in Japan; however, there were no outbreaks of *M. massiliense* in post-surgical infections and no suspicion of environmental contamination at four hospitals. Whilst the number of isolates in this study is limited, we infer that *M. massiliense* isolates in Clusters B and C may represent pathogenic strains spreading worldwide. In contrast, we infer that the Cluster D and E isolates from only Japanese patients are likely to be local strains. Further phylogenetic studies, including environmental strain collection and patient data, are needed to compare strains from other locations. In addition, further investigations using different molecular methods are required to clarify these hypotheses.

We consider that that 17-locus VNTR analysis with a higher level of HGDI was adequate for determining the distribution of *M. abscessus* complex. Interestingly, the highest overall 17-locus diversity (HDGI=0.977) exceeded that previously observed in the Malaysian isolates (0.9563) (Wong *et al.*, 2012). Compared with that determined using only six loci (0.9417), the diversity levels were increased, but the same discrimination level was attained using only six loci in a previous study (Wong *et al.*, 2012). We suggest that VNTR analysis is indispensable to allow refined molecular epidemiological screening to analyse the genetic distribution of *M. abscessus* complex. Additionally, the

VNTR analysis presented here showed that a single cluster was not formed by Japanese *M. abscessus* isolates featuring the C28 sequevar or by clarithromycin-acquired resistance isolates (Fig. 1). In Japan, C28 sequevar *M. abscessus* and clarithromycin-acquired resistance isolates contribute little to infection; therefore, the interpretation of these results has limitations.

When the clustering of isolates suggested by VNTR analysis was compared with PFGE patterns, we found that the two independent approaches were highly congruent for intra-species grouping. Our study also indicated that the clusters contained in the *hsp65* type I *M. massiliense* isolates were further divided by both methods. In particular, the *M. massiliense hsp65* type II-1 isolates were separated from the *M. abscessus* and *M. massiliense hsp65* type I isolates amongst the Japanese isolates. Moreover, the frequency of *M. massiliense hsp65* type II-1 was different from that in a neighbouring country. The distribution of *hsp65* type II-1 in our findings was lower (4/43; 9.3 %) compared with the higher reported distribution in South Korea (13/22; 59.1 %) (Jeon *et al.*, 2014). Hence, ethnic factors might also contribute to the susceptibility of a population to infection by different *hsp65* genotypes. However, these proportions might indicate that the prevalence of *M. massiliense hsp65* genotypes varies geographically. The recent publication of the complete genome sequence of a strain (Asan 50594) of *hsp65* type II also reveals its phylogenetic distinctness between *M. abscessus* complexes (Kim *et al.*, 2013). Further studies using global sample sets from other areas are needed to optimize the VNTR method and to compare *hsp65* type II-1 on a multi-region basis.

The results of our clarithromycin susceptibility tests were similar to previous results showing no significant differences between *hsp65* type I and type II (Jeon *et al.*, 2014; Kim *et al.*, 2013). However, the *M. massiliense* isolates with *hsp65* type II-1 always showed rough colonies. A previous study suggested that a virulence factor contributed to the capacity of rough *M. abscessus* complex strains to produce persistent pulmonary infections and it might be related to a higher virulence phenotype than the smooth type (Jönsson *et al.*, 2007). This issue should be investigated further because it could influence disease severity and clinical outcomes associated with clarithromycin susceptibility. We should conduct a further prospective research using additional serial isolates from the patients examined who were infected persistently.

In conclusion, the population structure amongst the 104 strains determined by 17-locus VNTR analysis revealed remarkable differences in *M. abscessus* and *M. massiliense* isolates. We also investigated *hsp65* genotypes (I and II-1) amongst the *M. massiliense* isolates and further different clusters amongst *M. massiliense hsp65* type I. The relatedness of the globally diverse *M. abscessus* complex, including the VNTR profiles of Malaysian isolates, was shown, but isolates with the *hsp65* type II-1 profile were not found in the Malaysian report (Wong *et al.*, 2012). Further studies with expanded populations are required in order to determine whether these clusters identify global commonality or local characterization of *M. abscessus* complex.

ACKNOWLEDGEMENTS

This work was supported by a Grant for a National Hospital Organization Respiratory Network Study 'Study of Respiratory Diseases (Tuberculosis, Lung Cancer, Diffuse Lung Diseases and Respiratory Insufficiency) using a Network of 54 Hospitals of National Chest Diseases in Japan' from the Ministry of Health, Labour and Welfare of Japan (Health Science Research grant H26-SHINKO-IPPAN-011) and MEXT/JSPS KAKENHI (grant 24590845). We are grateful to the members of the Clinical Research Center, Internal Medicine and Clinical Laboratory at National Hospital Organization Kinki-chuo Chest Medical Center for their cooperation and technical help.

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Tuberculosis 94 (2014) 649–656

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EPIDEMIOLOGY

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

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ARTICLE INFO

Article history:
Received 13 July 2014
Received in revised form
20 September 2014
Accepted 28 September 2014

Keywords:
Beijing genotype
Sublineage
Drug resistance
Genetic clustering
Vietnam
Variable number of tandem repeat

SUMMARY

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

1. Introduction

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

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

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http://dx.doi.org/10.1016/j.tube.2014.09.005

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

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

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

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

2. Materials and methods

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

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

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

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

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

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

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

2.3. Statistical analysis

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

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

3. Results

3.1. Characteristics of the study population

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

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

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

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

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

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

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

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

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

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

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

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

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

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

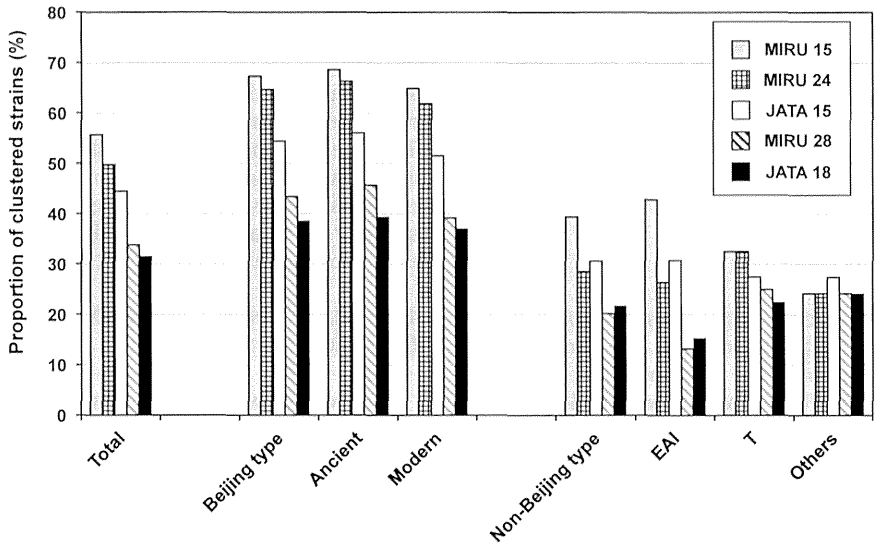

Figure 1. The proportion of clustered strains determined in each (sub)lineage using different VNTR locus sets. Using optimized-MIRU15, 24, 28, JATA15, and 18 locus sets, 465 MTB isolates were analyzed. The proportions of clustered strains were calculated.

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

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

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

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

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

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

3.6. Characteristics associated with ancient and modern Beijing sublineages

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

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

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

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

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

† No undernutrition indicates a body mass index over 18.5.

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

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

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

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

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

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

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

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

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

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

† No undernutrition indicates a body mass index over 18.5.

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments

We thank Ms. Akiko Miyabayashi for technical assistance and basic calculation of genotyping data.

Appendix A. Supplementary data

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

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 funders 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.

Ethical approval: This study was approved by the Ethical Committees of the Ministry of Health, Vietnam, National Center for Global Health and Medicine, and the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan.

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Human Immunology 75 (2014) 840–846

Contents lists available at ScienceDirect

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Age-dependent association of mannose-binding lectin polymorphisms with the development of pulmonary tuberculosis in Viet Nam

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ARTICLE INFO

Article history:
Received 15 January 2014
Accepted 7 June 2014
Available online 19 June 2014

Keywords:
Pulmonary tuberculosis
Latent tuberculosis infection
Mannose-binding lectin
Polymorphism
Genetic association

ABSTRACT

Mannose-binding lectin (MBL) binds to pathogens and induces complement-mediated opsonophagocytosis. Although the association between *MBL2* polymorphisms and tuberculosis (TB) has been studied in various populations, the results are controversial. We explored the stages of TB associated with *MBL2* polymorphisms. *X/Y* (rs7096206) and *A/B* (rs1800450) were genotyped in 765 new patients with active pulmonary TB without HIV infection and 556 controls in Hanoi, Viet Nam. The *MBL2* nucleotide sequences were further analyzed, and plasma MBL levels were measured in 109 apparently healthy healthcare workers and 65 patients with TB. Latent TB infection (LTBI) was detected by interferon-gamma release assay (IGRA). The *YA/YA* diplotype, which exhibited high plasma MBL levels, was associated with protection against active TB in younger patients (mean age = 32) ≦ 45 years old (odds ratio, 0.61; 95% confidence interval, 0.46–0.80). The resistant diplotype was less frequently found in the younger patients at diagnosis ($P = 0.0021$). *MBL2* diplotype frequencies and plasma MBL levels were not significantly different between the IGRA-positive and -negative groups. *MBL2 YA/YA* exhibited a protective role against the development of TB in younger patients, whereas the *MBL2* genotype and MBL levels were not associated with LTBI. High MBL levels may protect against the early development of pulmonary TB after infection.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB) in humans and presumably infects a third of the world's population. *Mtb* establishes a persistent infection in immune cells such as macrophages, and 5–10% of immunocompetent individuals develop active TB during their lifetime, whereas the others limit infection by successful containment of *Mtb* in granulomas. The innate immune response induces activation of the T helper 1 (Th1)-type immune system and plays an important role in host defense against the development of TB [1]. Many studies have reported the association between TB and polymorphisms of host genes related to innate immunity [2].

Mannose-binding lectin (MBL) is an acute-phase serum protein in the collectin family that recognizes a pathogen by its carbohydrate-recognition domains [3]. MBL is synthesized in the liver and circulates in the form of oligomers complexed with MBL-associated serine proteases (MASPs). Upon binding to the sugar moieties on the pathogen surface, MASPs are activated to initiate the lectin pathway of complement activation, which results in opsonization and phagocytosis or lysis of microorganisms. Besides its direct action as an opsonin and its key role in the lectin pathway, MBL may modulate inflammatory responses and immune activation [4].

Abbreviation: Mtb, Mycobacterium tuberculosis.

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http://dx.doi.org/10.1016/j.humimm.2014.06.006
0198-8859/