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–I was low (9.3 %), *M. massiliense* hsp65 type II–I 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.

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,
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 sequenvar) 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-choo 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 Fukuijui 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-choo 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  ermF (5’-GACCGGG-GGCTTCTTGTGAT-3’) and  ermR1 (5’-GACTTCCCCGCCACCA-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 *Hinfl* 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. Thioura (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 Drai (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 *Hinfl* 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 (≤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* JCM153569T. We identified four further 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

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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 subdivided 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
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

<table>
<thead>
<tr>
<th>Species</th>
<th>Japan</th>
<th>Malaysia</th>
<th>Reference strain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. abscessus</em></td>
<td>59</td>
<td>10</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td><em>M. massiliense</em></td>
<td>43</td>
<td>24</td>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td><em>M. bolletii</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

**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*. 

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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

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**Fig. 3.** Comparison between the PFGE dendrogram produced using *DraI* 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.
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 I-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.

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