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Research paper

Phylogenetic uniqueness of *Mycobacterium avium* subspecies *hominissuis* isolated from an abnormal pulmonary bovine case

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ABSTRACT

Mycobacterium avium subspecies *hominissuis* (MAH) is an important cause of infection in human pulmonary and swine intestinal cases. Although MAH is isolated from environmental sources frequently, infections of other animals have rarely been analysed. Recently, we detected granulomatous inflammation in bovine lung as an abnormal postmortem inspection case. To ascertain its genetic profile, we conducted a variable numbers of tandem repeats (VNTR) analysis and genomic characterization using deep sequencing. The VNTR type was a unique profile that differed from reported genotypes, but it was assigned within a broad genotypic complex of isolates from human patients and bathrooms. Genomic comparison with 116 registered genome sequences of the subspecies revealed that the strain was separate from five major genetic population groups proposed previously. Although the infection source remains unclear, its isolation from various resources such as animal infection cases should be elucidated more extensively to reveal its genetic diversity and ecological context.

1. Introduction

Mycobacterium avium complex (MAC) is a group of related opportunistic pathogens comprising *Mycobacterium avium*, *Mycobacterium intracellulare*, and several less commonly encountered species which cause human disease. MAC pathogens have been isolated from various animal hosts and environmental sources. Recently increased numbers of cases of *M. avium* infections has led to awareness of the pathogen as a global public health concern (Biet et al., 2005; Ignatov et al., 2012; Turenne et al., 2007; Winthrop, 2010). The infection, which frequently occurs in immunocompetent individuals, generally manifests as a slowly progressive, often debilitating, lung disease (Griffith et al., 2007).

Mycobacterium avium subspecies *hominissuis* (MAH) is a subspecies comprising *M. avium* with three other subspecies: *M. avium* subspecies *paratuberculosis* (MAP), *M. avium* subspecies *avium* (MAA), and *M. avium* subspecies *silvaticum* (MAS) (Rindi and Garzelli, 2014). Of them, MAP is the most notable pathogenic subspecies, causing Johne's disease in livestock: a chronic progressive contagious granulomatous enteritis that principally affects ruminants. Another subspecies, MAA is considered as an agent of avian tuberculosis. Although MAS has not been isolated from the environment, it also causes avian tuberculosis (Dvorska et al., 2003). In contrast to the other subspecies, MAH has been regarded as an environmental opportunistic pathogen affecting humans and swine worldwide (Falkinham III, 2009). In addition to typical hosts, the subspecies has been isolated from other animals such as cattle, dogs, birds, deer, and horses (Mijs et al., 2002; Thorel et al., 1997). Despite frequent infection of the two natural hosts, human and pigs, their respective ordinary sites of infection differ: lungs and intestinal tracts. The reasons remain unknown, but differences of their major

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transmission resources (airborne and faecal–oral) have been inferred as a reason (Agdestein et al., 2014). Soil and water (drinking water and tap aerosols) are regarded as natural sources of MAH infection in humans (Falkinham III, 2003; von Reyn et al., 1994). The possibility that MAH has evolved as a zoonotic pathogen, transferred from animals to humans (Haist et al., 2008), remains to be verified.

Multilocus variable numbers of tandem repeats (VNTR) analysis is a highly discriminable typing method that has been applied to several mycobacterial species as a genotyping method (Shin et al., 2013; Supply et al., 2006). This typing method has already been used for MAH isolates (Ichikawa et al., 2015; Inagaki et al., 2009; Iwamoto et al., 2012; Thibault et al., 2007). In an earlier study, no VNTR profile of swine isolates was found to be identical to those of isolates from either human patients or bathrooms (Iwamoto et al., 2012). Their VNTR types revealed that the respective sources (swine vs. human and bathrooms) are in two separate genetic clusters, which suggests that they have diverged according to the disseminated infection in swine and pulmonary infection in human.

In addition to genotyping methods using polymorphic alleles, high-throughput sequencing techniques such as Illumina sequencers have been used recently for molecular epidemiological investigation of MAH (Uchiya et al., 2017; Yano et al., 2017). Powerful outputs can provide large amounts of data for almost all genomes of microbial strains, thereby supporting elaborate phylogeny and population genetics of pathogenic bacteria. For instance, Uchiya et al. (2017) analysed various clinical strains from patients with progressive and stable diseases to identify genetic groups of MAH causing each infection prognosis. Yano et al. (2017) also specifically examined the description of the genetic variety and population structure of MAH. Their observations related to MAH were mostly consistent: they found mainly three to five clusters, two of which were occupied by isolates from East Asia (mainly Japan). These East Asian clusters, designated as I-a/MahEastAsia2 and I-b/MahEastAsia1, possess common accessory genes shared by ancestral or recent horizontal recombination (Yano et al., 2017). However, I-a/MahEastAsia2 has a specific genomic element that might be responsible for its high virulence (Uchiya et al., 2017).

These genomic approaches have led to profound inferences related to the ecology and evolutionary processes of MAH as a pathogen. Nevertheless, intensive studies of MAH have been restricted to clinical strains isolated from human patients. In this regard, the genetic diversity of MAH has been unveiled in detail yet.

This report describes a unique pulmonary case of bovine MAH infection. We isolated the causative strain and identified its genotype based not only on VNTR typing but also on results of deep sequencing analysis. The genotype was then compared with those of strains isolated from various sources, which was helpful to understand the ecological context and broad genetic diversity of MAH.

2. Material and methods

2.1. MAH Identification

Lesion-containing pulmonary tissue from slaughtered cattle was homogenized and cultured with Ogawa Slant Medium (Serotec Co. Ltd., Tokyo, Japan) at 35 °C for six weeks. Microscopic smears of the cultured bacilli were stained using Ziehl–Neelsen (ZN) method. The isolate was designated as BVLA01. To ascertain the species, we used DDH Mycobacteria Kit (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). The commercial kit was based on DNA-DNA Hybridization (Kusunoki et al., 1991). We used a 16S rRNA sequencing (Devulder et al., 2005) to identify the colonies. For DNA extraction, a loopful of colonies from the strain was suspended in TE buffer (300 µl) and heat-killed in a boiling water bath for 15 min. The crude supernatant was used in PCR amplification. For subspecies identification, PCR amplicons of the *hsp65* gene were analysed by sequencing according to a method described elsewhere (Turenne et al., 2006). The PCR products were sequenced using the Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA) on a genetic analyzer (AB 3500; Applied Biosystems).

2.2. Histopathological analysis of tissues

Lesion-containing tissues were completely excised and used for histopathological diagnosis. The excised mass was processed according to a standard protocol: after it was fixed in neutral-buffered formalin (10%, vol/vol) and embedded in paraffin, sections (3–5 µm) were cut and stained with haematoxylin and eosin (HE) for histological examination, and with ZN stain to detect acid-fast bacilli.

2.3. VNTR genotyping

For VNTR genotyping, 19 loci were examined, covering two VNTR subsets used in previous reports: seven loci of representative mycobacterial interspersed repetitive units (MIRU)-VNTR (Thibault et al., 2007) and 14 loci of *M. avium* tandem repeat (MATR)-VNTR (Ichikawa et al., 2015) (Table 1). Then PCR was conducted using TaKaRa ExTaq (0.125 U/reaction), dNTPs (0.2 mM), and GC buffer I (Takara Bio Inc., Shiga, Japan). The PCR cycling parameters were the following: 94 °C for 3 min followed by 30 cycles of amplification (94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s), with final extension at 72 °C for 3 min. Each PCR product was analysed (QIAxcel; Qiagen Inc., Hilden, Germany) and subjected to agarose gel electrophoresis to ascertain the accurate size of the PCR products. A DNA screening kit 2400 (Qiagen Inc.) was used for electrophoresis in the QIAxcel system, according to the manufacturer's recommendations. The VNTR unit copy numbers for each of the 19 polymorphic loci were assigned based on the respective amplicon sizes and were calculated based on in silico analysis of the complete genome sequence of the reference strain *M. avium* 104 (GenBank accession number NC_008595.1).

Clustering analysis of VNTR profiles was performed based on the minimum spanning tree (MST) algorithm using the genotype of BVLA01 and previously reported isolates of various origins using software (Bionumerics v.4.6; Applied Maths Inc., St., Martens-Latem, Belgium). Based on the data, respective subsets of loci were selected for comparing the BVLA01 genotype with the reported data: seven MLVA loci were selected for comparison with VNTR profiles of isolates from various sources (Radomski et al., 2010; Tirkkonen et al., 2010; Iwamoto et al., 2012). To evaluate the genetic relatedness between the populations of clinical MAH isolates from different geographic regions (Ichikawa et al., 2015), 14 MATR-VNTR loci were selected and analysed.

2.4. Genomic comparison

Short reads of BVLA01 genome were sequenced using MiSeq (Illumina, Inc., San Diego, CA). Briefly, a TruSeq Nano DNA Library Preparation Kit was used with MiSeq Reagent Kit v3 (600 cycle) for sequencing. The reads can be retrieved under DDBJ BioProject ID: PRJDB6614. They were assembled using CLC Genomics Workbench (ver. 9.5.2) to 93 scaffolds longer than 500 bp. In all, the length was 5,233,201 bp (The longest scaffold was 476,069 bp). The shortest sequence length at 50% of the genome (N50) was 168,380 bp.

All 116 genome sequences of MAH registered as MAH by November 2017 were retrieved from the International Nucleotide Sequence Database (INSD) via GenBank/NCBI (Table S1). All sequences were annotated with PROKKA (ver. 1.11) to estimate coding genes (Seemann, 2014). Orthologous genes were searched using get_homologues (ver. 02032017) (Contreras-Moreira and Vinuesa, 2013). Strain 104 was determined as a reference. The condition was set strictly (% length difference within clusters = 100) to facilitate subsequent analyses, which provided 436 nucleotide sequence clusters with identical lengths among 7 complete genomes and 110 draft sequences. Two of

Table 1
VNTR profiles for *M. avium* subsp. *hominissuis* strain Bvla01 and similar types[a] found in previous studies.

Strain Name	No. of locus variation	MATR-VNTR (14-locus)										
		MATR-1	MATR-2[b] MIRU-292	MATR-3[b] MIRU-X3	MATR-4	MATR-5	MATR-6	MATR-7	MATR-8	MATR-11	MATR-12	MATR-13
Bvla01	0	2	1	1	3	2	2	2	2	2	3	1
753MA	4	2	1	1	2	2	2	2	2	2	3	2
875MA	4	2	1	1	2	2	2	2	2	2	3	2
390MA	5	2	1	1	2	2	2	1	2	2	3	2
HP64	5	2	1	5	2	2	2	2	2	2	3	2
422MA	5	2	1	1	2	2	2	2	2	2	3	0
546MA	5	2	0	1	2	2	2	2	2	2	3	2
OCU682	5	2	0	1	2	2	2	2	2	2	3	2
OCU432	5	2	0	1	2	2	2	2	2	2	3	0
OCU433	5	2	0	1	2	2	2	2	2	2	3	0
504MA	5	2	0	1	2	2	2	2	2	2	3	0
104	12	2	2	5	2	3	3	3	4	5	3	2

Strain Name	MATR-VNTR (14-locus)			MIRU-VNTR (7-locus)						
	MATR-14	MATR-15	MATR-16	MIRU-07	MIRU-10	MIRU-25	MIRU-32	MIRU-47	MATR-2[b] MIRU-292	MATR-3[b] MIRU-X3
Bvla01	2	2	1	1	5	4	8	3	1	1
753MA	2	2	3	1	3	4	8	3	1	1
875MA	2	2	3	1	3	4	8	3	1	1
390MA	2	2	3	1	3	4	8	3	1	1
HP64	2	2	1	1	3	4	7	3	1	5
422MA	2	2	3	1	2	3	8	3	1	1
546MA	2	2	1	1	2	3	8	3	0	1
OCU682	2	2	1	1	2	3	8	3	0	1
OCU432	2	2	1	1	2	3	8	3	0	1
OCU433	2	2	1	1	2	3	8	3	0	1
504MA	2	2	3	1	2	4	8	3	0	1
104	4	4	3	1	2	1	7	3	2	5

[a] Top 10 similar profiles in comparison of all 19 loci.
[b] Two loci were used both in MATR-VNTR and MIRU-VNTR, with different synonyms.

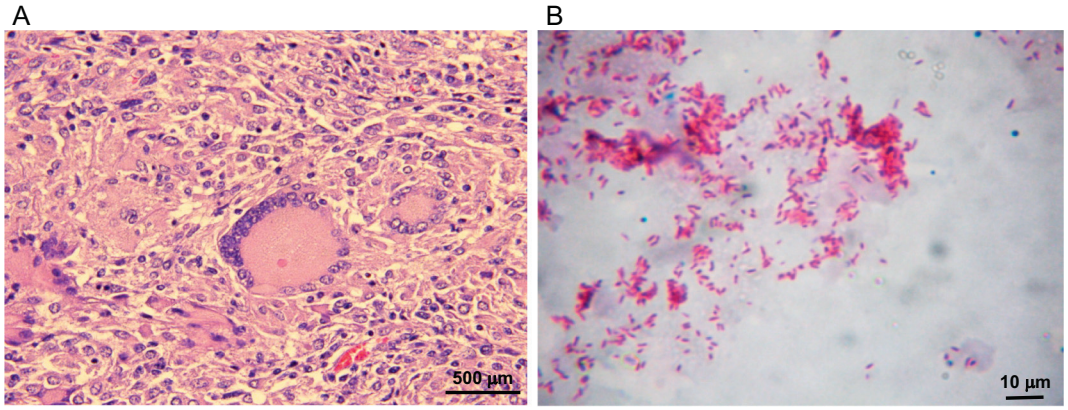


Fig. 1. Histological evaluation of the infected bovine tissues and the isolated bacteria. A: Severe multifocal to diffuse histiocytic inflammation and multinucleated giant cells were observed in the lungs during microscopic examination after haematoxylin and eosin (HE) staining. Moderate diffuse interstitial to granulomatous pneumonia was apparent. The inset shows a giant cell within an inflammatory lesion. The alveoli were filled with foamy macrophages and epithelioid cells. B: Acid-fast bacilli were isolated from tissue specimens, cultured, and stained using Ziehl–Neelsen (ZN) method.

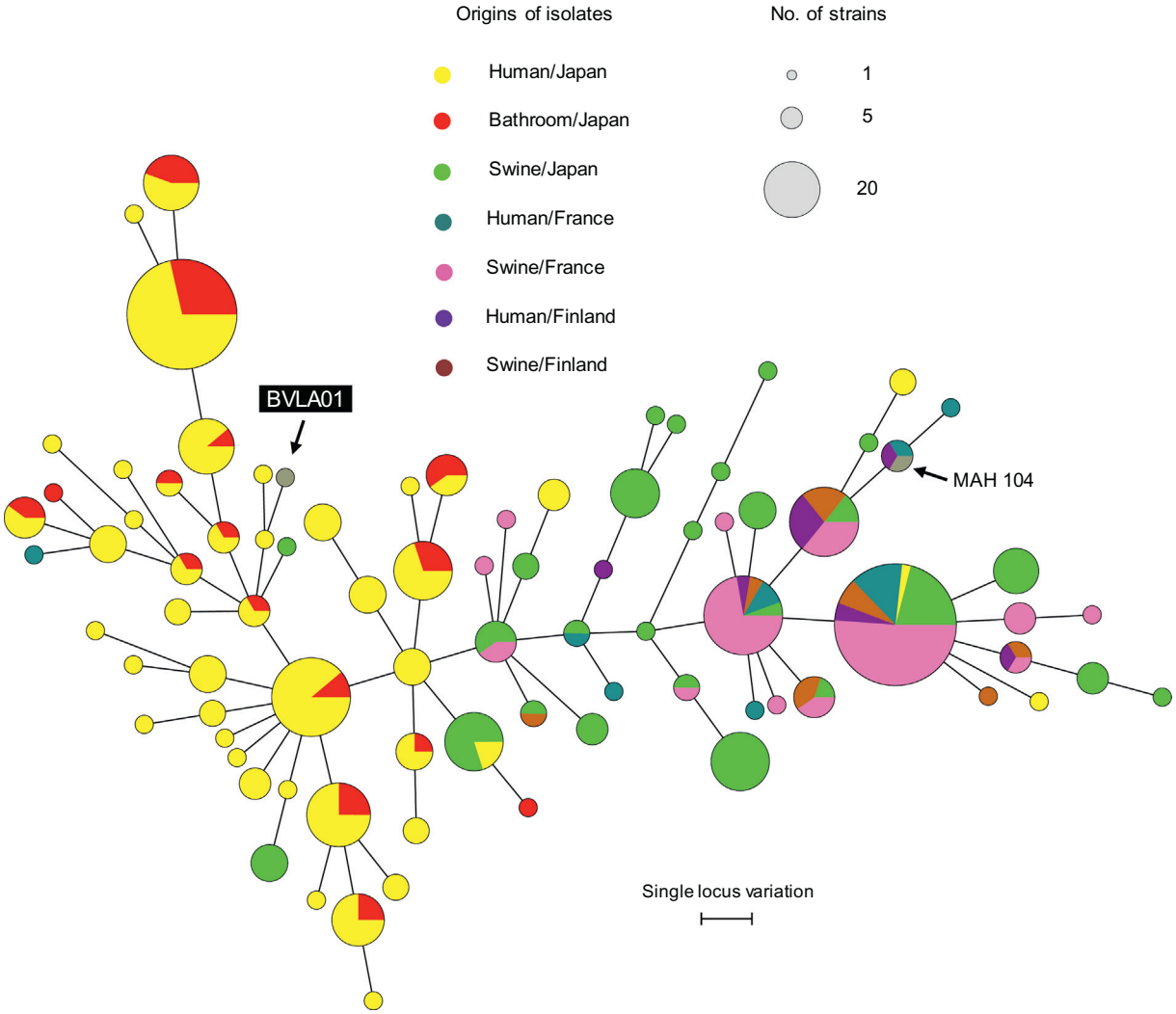


Fig. 2. Combined minimum spanning tree (MST) analysis based on 7-loci (MIRU-07, -10, -25, -32, -47, -292, and -X3) MIRU-VNTR genotyping of MAH strains. Bovine MAH strain isolated in the current study and previously published strain data were used for the analysis. Circle sizes are proportional to the number of isolates sharing an identical VNTR pattern. Strains were isolated from human hosts (Japan, yellow; France, blue green; and Finland, purple), bathrooms (Japan, red), and swine (Japan, green; France, pink; and Finland, brown). MAH 104, a MAH reference strain, and BVLA01 are shown in grey. Lines connecting two types denote single- or double-locus variants with each length. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

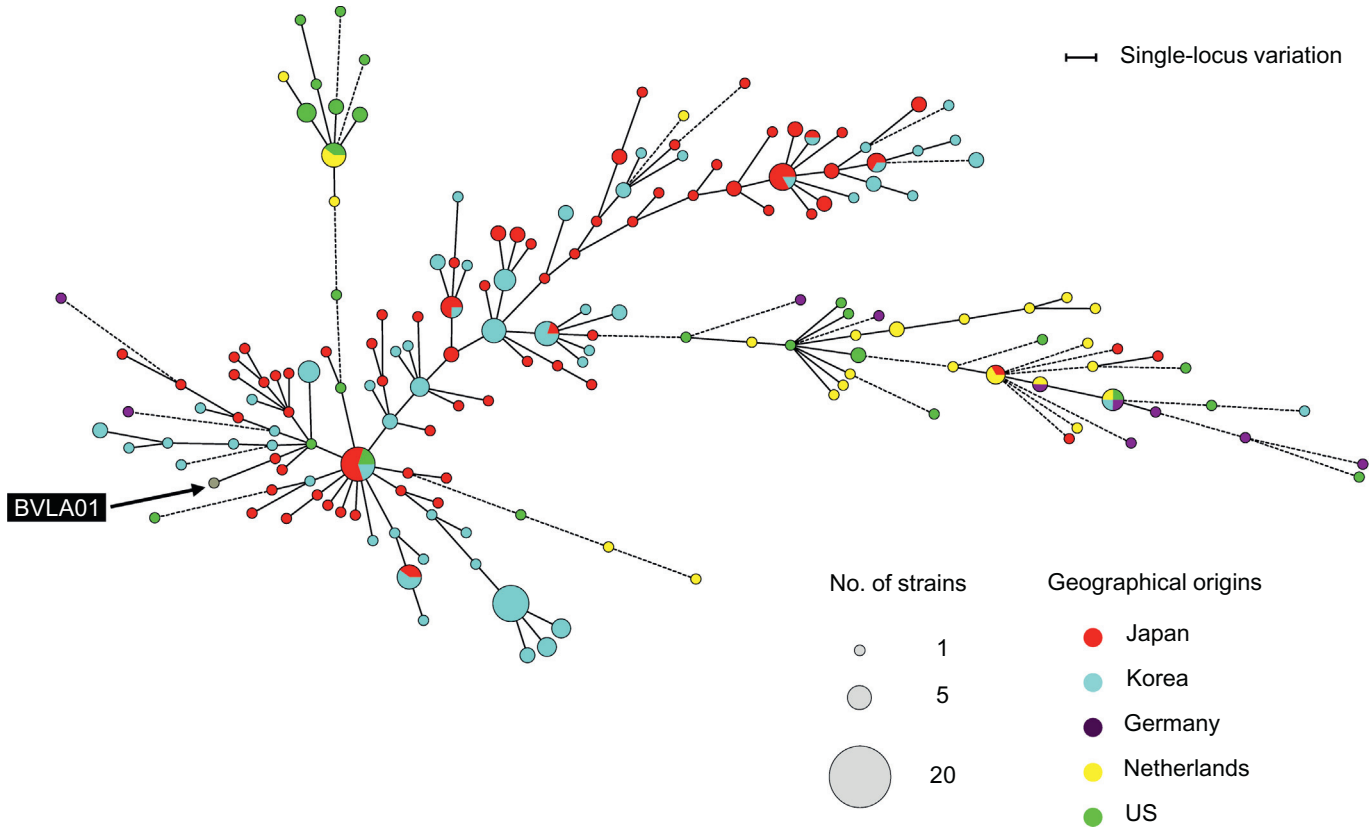


Fig. 3. Minimum spanning tree (MST) analysis based on 14-locus MATR-VNTR genotyping of 261 MAH strains from different geographic regions. Circles correspond to different VNTR genotypes. Circle sizes are proportional to the number of isolates sharing an identical pattern. Strains were isolated from Japan (red), Korea (blue), the United States (green), the Netherlands (yellow), and Germany (purple). Strain BVLA01 is shown in grey. Solid lines connecting two types denote single- or double-locus variants with each length. Dotted lines connect more than three-locus variants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

them were removed because they had ambiguous nucleotides and an unreliable match. Finally, 434 gene-coding sequences (271,344 bp) including 7011 polymorphic nucleotides were concatenated and aligned using MAFFT (ver. 7.245) (Katoh and Standley, 2013).

Alignment was used for phylogenetic tree and network construction. A neighbour-joining tree was constructed with 500 bootstrap iterations using MEGA7 (Kumar et al., 2016). The evolutionary distances were computed using the maximum composite likelihood method. The rate variation of nucleotide substitution among sites was modeled with a gamma distribution (parameter = 5). SplitsTree 4 was used to construct a phylogenetic network (Huson and Bryant, 2006). The JC69 model was incorporated for calculations.

3. Results

3.1. Pathological observations and bacterial identification

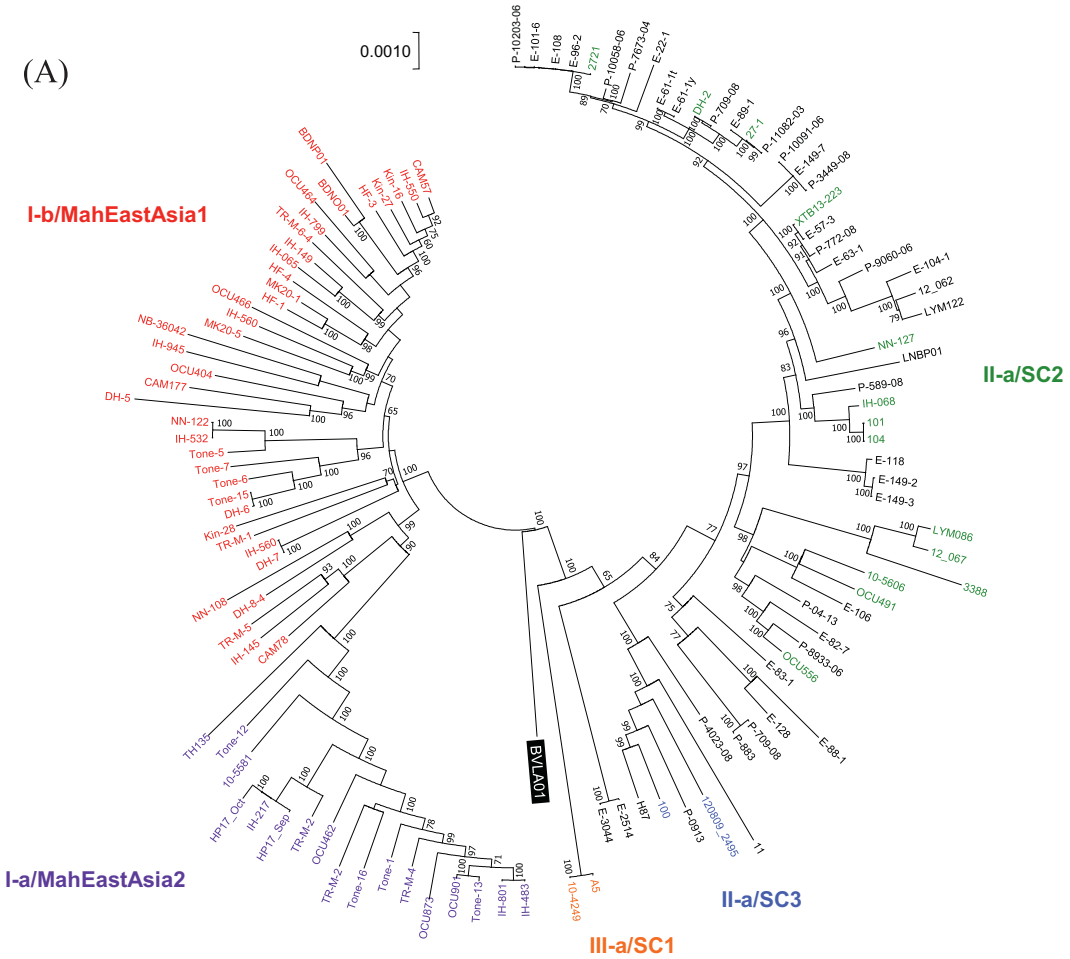
A pulmonary mycobacterial infectious lesion was detected in a neutered 25-month-old bullock for postmortem examination of meat inspection findings at the Institute of Social Welfare and Public Health (Nishi-Kasugai, Aichi, Japan) in September 2014. No sign of immunosuppression or episode of dyspnoea was observed.

As revealed by necropsy, most gross lesions were located in the lung of the infected bovine. The lesions had greyish-white nodules of various sizes. Large nodules in the lung were irregularly thickened and exhibited a rough surface. Lymph nodes of the cervical, bronchial, and mediastinal regions were enlarged but they contained no tumour. No lesion was localized in the wall of the intestine; such lesions would be typical of MAP infection (Charavaryamath et al., 2013; Koets et al., 2015). Furthermore, no thickening of the intestinal mucosa or mesenteric lymph node enlargement was apparent: no leukocyte, epithelioid cell, or giant cell infiltrate of the intestinal wall was detected by histologic examination.

The pulmonary granulomas were observed microscopically after HE staining (Fig. 1-A). Histologic examination of the lung revealed granulomatous pneumonia with macrophages and epithelioid cells. Marked intraalveolar and parenchymal infiltrations of foamy histiocytes were observed, in addition to severe multifocal to diffuse histolytic inflammation with multinucleated Langerhans giant cells (Fig. 1-A). ZN staining of lung lesion resulted in no acid-fast bacilli. After 14 days of culture of lung lesion homogenates, bacterial growth was observed on solid media. Acid-fast bacilli from isolates were observed microscopically after ZN staining (Fig. 1-B). The isolate was identified as *M. avium* based on DDH Mycobacteria Kit analysis and 16S rRNA gene sequencing (data not shown). Further analysis was conducted to identify the subspecies using direct sequence of the 3′-fragment of *hsp*65. The clinical strain, named BVLA01, was finally identified as MAH with *hsp65* sequevar code 1 (Iwamoto et al., 2012; Turenne et al., 2006).

3.2. VNTR genotyping

The 19-locus VNTR genotype of BVLA01 strain was determined, which covered two VNTR subsets used in previous reports (7 loci of representative MIRU-VNTR and 14 loci of MATR-VNTR) (Table 1). Each subset of VNTR genotypes was then compared with 349 genotypes from various sources (pigs, the environment, and human patients) and with 261 genotypes from different geographical regions (East Asia, Europe, and the United States) described in previous reports (Iwamoto et al.,



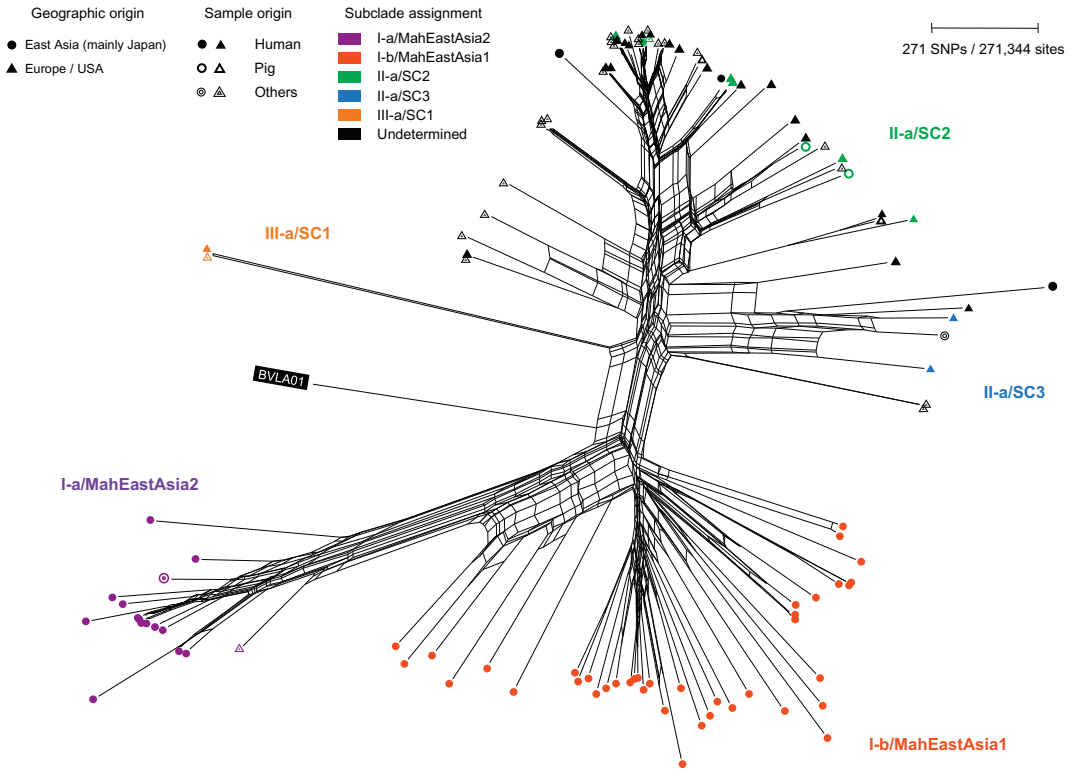


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Fig. 4. Phylogenetic assignment of BVLA01 with registered genome sequences of 116 MAH strains using nucleotide sequences of 434 genes. (A) An unrooted neighbour-joining tree was constructed. Percentages of replicate trees in the bootstrap test were shown next to the branches when they were over 60. Five subclusters reported by two earlier studies of comparative genomics of MAH (Uchiya et al., 2017; Yano et al., 2017), I-a/MahEastAsia2, I-b/MahEastAsia1, II-a/SC2, II-a/SC3, and III-a/SC1, were estimated in this tree. Strains that have been assigned already to the subclusters were coloured with the same colours as those used for subclusters. (B) A phylogenetic network of the 117 MAH strains. Geographic and sample origins were designated by each symbol as shown. Subcluster assignment was indicated by colours as in (A).

2012; Ichikawa et al., 2015) (Table S2). In both comparisons, the genotype of BVLA01 was a unique orphan type. When the BVLA01 genotype was mixed into a VNTR-based clustering analysis with strains from various sources, BVLA01 was located near isolates from humans and bathrooms (Fig. 2). BVLA01 and the isolates from East Asia (Japan and Korea) localized closely in MST analysis of 14-locus MATR-VNTR, indicating a high degree of relatedness (Fig. 3). In spite of the high relatedness, BVLA01 exhibited constant difference in three loci (MATR-4, MATR-13, and MIRU-10), as its unique character, with similar profiles found in the previous VNTR data (Table 1).

3.3. Genomic comparison

To clarify the phylogenetic distance between BVLA01 and other isolates from various origins, its draft genome sequence was compared with registered sequences. Scaffolds of BVLA01 and 116 draft or complete genome sequences of MAH strains retrieved from INSD were (re-) annotated using a bioinformatic tool (PROKKA; Seemann, 2014). Using stringent conditions for gene clustering, after 434 genes common to the 117 genomes were selected as a core gene group, they were used for phylogenetic analysis (Fig. 4). The genes are presented in Table S3. Five subclusters reported by Yano et al. (2017) were observed clearly in the tree (A) and the network (B). Strains in East Asia (mainly Japan: 60 of 62 strains) occupied two subclusters, I-a/MahEastAsia2 and I-b/MahEastAsia1, whereas strains from Europe/USA were found predominantly in the other subclusters (II-a/SC2 and SC3, and III-a/SC1). The subdivision was comparable with VNTR clustering analysis in Fig. 3. In spite of close relatedness with human strains in Japan by VNTR clustering (Fig. 3), BVLA01 was separate from all subclusters in the phylogenetic tree and network (Fig. 4).

4. Discussion

The bovine case of BVLA01 in this study was categorized symptomatically as a lung infection (Fig. 1), presenting the possibility of airborne transmission, similarly to a typical human infection. It was unusual for MAH to be isolated from pulmonary granulomatous tissues in animals. Generally, MAH not only causes infections in human and animals; it also inhabits the environment (Rindi and Garzelli, 2014). Recently, genetic diversity, population structure, and the relationship between its genotypes and infection prognosis were scrutinized in earlier studies (Uchiya et al., 2017; Yano et al., 2017). Regarding relations between human and animal infection, high similarity between MAH isolates from pigs with disseminated disease and from clinical human sputa in European countries has been reported (Radomski et al., 2010; Tirkkonen et al., 2010), which suggested the existence of epidemiological links between human and animal infections (Johansen et al., 2007; Mobius et al., 2006; Pate et al., 2008; Tirkkonen et al., 2010). In contrast to it, the relatedness between human MAH isolates and swine isolates was low in Japan (Iwamoto et al., 2012), which was contradictory to results obtained for European countries. Genotyping methods have been used as an epidemiological tool to unravel epidemiological traits and sources of MAH infections (Alvarez et al., 2011; Pate et al., 2011; Radomski et al., 2010; Thibault et al., 2007). Continuous accumulation of genotype data of MAH strains from various origins will be necessary to provide us with a better understanding of their transmission, constituting a highly reliable risk assessment for the prevention of endemic spread of the species.

In this study, we attempted to infer genetic relatedness between BVLA01 and registered data of MAH strains using two methods: VNTR typing and phylogenetic analysis based on genome sequences. Subdivision of VNTR clusters between East Asian and European/US strains (Fig. 3) was apparently comparable with phylogeny based on genomic comparison (Fig. 4-B). In the phylogenetic network, two strains from pigs in Japan belonged to II-a/SC2 (Fig. 4-B), which seemed consistent with VNTR clustering with those of European/US strains (Fig. 2). When the VNTR genotype of BVLA01 was projected onto the clustering analysis of a population of strains, it was classed into a cluster composed of strains from human patients and bathrooms (Fig. 2). It is noteworthy that genomic comparison revealed that the phylogenetic position of BVLA01 was not similar to that of any other strain (Fig. 4), in contrast to the results of VNTR analysis. This result suggests that VNTR clustering might be insufficient to infer the phylogeny of MAH strains.

The phylogenetic uniqueness of BVLA01 underscores the lack of knowledge available in relation to the genetic diversity of MAH. Our simple methods for phylogenetic tree construction using strict orthologous clusters (434 genes) were useful to classify MAH strains into subclusters properly (Fig. 4). The MAH subclusters were observed based mainly on human clinical strains. Therefore, an authentic genetic population structure of MAH might be underestimated because of a lack of data from various sources. Actually, MAH was characterized by its broad habitat range, which might be conducive to mutual recombination (Yano et al., 2017). Systematic strain collection and genomic comparison that includes samples from animals and the environment will be necessary to clarify the whole picture of genetic diversity, adaptation strategy, and transmission pathways of MAH.

Before analysis, it was expected that putative transmission routes of BVLA01 could be estimated according to genetic similarity with other strains, but it ended in failure because of its phylogenetic uniqueness (Fig. 4). The bovine infection was a solitary case, which caused no subsequent outbreaks. Epidemiologic approaches yielded no clue its infection sources. Generally, the endemic state of MAH in wildlife and ruminant livestock is unclear: in contrast with multiple MAP surveillance trials (Pruvot et al., 2013; Waddell et al., 2016), the prevalence of MAH in wildlife and the environment has not been surveyed widely. To make MAH surveillance more effective in future, various animals and environments must be considered as potential sources of infections of MAH, unlike the host-adapted pathogens like MAP. This is true because the style of transmission and disease spread might lead to atypical organs being infected and atypical symptoms arising, as observed in the bovine case described herein. Intensive collection of clinical strains from infected animals for comparison with environmental isolates is important for the re-evaluation of their potential as pathogenic agents. Efficient collection of clinical strains is expected to be crucially important for obtaining helpful insight not only to ascertain appropriate disease control in the veterinary field but also in the context of human health based on a one-health approach.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.04.013.

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