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Molecular typing and genetic characterization of *Mycobacterium avium* subsp. *hominissuis* isolates from humans and swine in Japan

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Mycobacterium avium subsp. *hominissuis* (MAH) causes disease in both humans and swine; however, the genetic variations in MAH isolates are unclear. The aim of this study was to elucidate the genetic variations in MAH isolates from humans and swine in Japan. We analysed the 16S–23S rDNA internal transcribed spacer (ITS) sequence and variable number of tandem repeats (VNTRs) using the *Mycobacterium avium* tandem repeat loci, prevalence of IS*Mav6* and clarithromycin resistance for MAH isolates from patients with pulmonary MAC (pMAC) disease (n=69), HIV-seropositive and blood-culture-positive (HIV-MAC) patients (n=28) and swine (n=23). In the minimum spanning tree based on VNTR analysis, swine MAC isolates belonged to a cluster distinguishable from that of human pMAC isolates. Isolates from HIV-MAC were scattered throughout both clusters. The major three distinct sequevars, Mav-A, Mav-B and Mav-F, were determined according to 16S–23S rDNA ITS sequence analysis in addition to three new sequevars, Mav-Q, Mav-R and Mav-S. Mav-A and Mav-F comprised the majority of human pMAC strains; in contrast, Mav-B predominated in swine isolates. Distribution of ITS sequevars in the minimum spanning tree based on VNTR analysis showed similar clusters of isolates from different origins, i.e. human pMAC, HIV-MAC and swine. These results, together with IS*Mav6* possession and clarithromycin resistance, revealed genetic diversity of MAH strains recovered from humans and swine. Molecular epidemiology and genetic characterization in the present study showed the distinctive genetic evolutionary lineage of MAH strains isolated from human pMAC diseases and swine.

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INTRODUCTION

Mycobacterium avium is ubiquitous in the environment such as soil and water, including drinking water (Biet *et al.*, 2005; Falkinham *et al.*, 2015). *M. avium* is composed of four subspecies, i.e. *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* (MAH). These subspecies represent distinct organisms, each endowed with specific

Abbreviations: CLA, clarithromycin; *hsp65*, heat shock protein 65; ITS, internal transcribed spacer; MAC, *Mycobacterium avium* complex; MAH, *Mycobacterium avium* subsp. *hominissuis*; MATR-VNTR, *Mycobacterium avium* tandem repeat-variable number of tandem repeat; MST, minimum spanning tree; pMAC, pulmonary *Mycobacterium avium* complex; SNP, single nucleotide polymorphism.

pathogenetic and host range characteristics. The existence of insertion sequences (i.e. IS*900*, IS*901* and IS*1245*) can be used to distinguish subspecies (Turenne *et al.*, 2007); however, one of the housekeeping genes of *M. avium*, the heat shock protein 65 (*hsp65*) sequence, has been shown to provide more detailed information for subspecies identification (Turenne *et al.*, 2006). MAH, one of the species of *Mycobacterium avium* complex (MAC), is a pathogenic organism which can infect both human and pigs. It causes not only disseminated diseases in patients with human immunodeficiency virus infection but also pulmonary disease even in immunocompetent patients (Turenne *et al.*, 2006), and the incidence of pulmonary MAC (pMAC) infection is increasing in Japan (Ide *et al.*, 2015).

A variable number tandem repeat (VNTR) typing method using the *M. avium* tandem repeat loci (MATR-VNTR) has been used in Japan for epidemiological studies of clinical isolates of MAH. MATR-VNTR typing is inexpensive, easy to perform and universally comparable and has an excellent discriminatory power – a Hunter–Gaston discriminatory index of 0.990 for MAH has been reported (Inagaki *et al.*, 2009). In addition, the molecular epidemiology of MAH has been studied using the 16S–23S rDNA internal transcribed spacer (ITS) sequence (Mijs *et al.*, 2002; Novi *et al.*, 2000). According to this typing system, strains with a given ITS sequence are grouped into a specific 'sequevar'. Seven *M. avium* sequevars, named Mav-A to Mav-G, have been reported so far (Frothingham *et al.*, 1993; Mijis *et al.*, 2002; Murcia *et al.*, 2006; Novi *et al.*, 2000; Stout *et al.*, 2008) (Table 1). Using VNTR analysis, Ichikawa *et al.* (2015) showed genetic diversity of MAC isolates, i.e. MAH and *Mycobacterium intracellulare*, causing pulmonary diseases recovered from different geographical regions, such as Japan, South Korea, The Netherlands, Germany and USA. Iwamoto *et al.* (2012) also revealed genetic similarity of not only MAH strains isolated from humans and bathroom in Japan but also MAH strains from pigs in Japan and humans and pigs in France and Finland. Iakhiaeva *et al.* (2016) showed the variation of 16S–23S rDNA ITS sequevars of MAH isolates from respiratory and household water biofilm samples in the USA and also showed the difference in the distribution of copy numbers of tandem repeats, not in the range of copy numbers by the detailed analysis of VNTR types of isolates from USA and Japan.

In the present study, we elucidated the genetic diversity of MAH strains isolated from human patients with pMAC disease and HIV-seropositive disseminated disease and pigs using 16S–23S rDNA ITS analysis, MATR-VNTR typing, IS*Mav6* possession and clarithromycin (CLA) resistance, which have important clinical relevance.

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METHODS

Bacterial strains. *M. avium* strain 104 (GenBank accession no. NC008595), *M. avium* subsp. *avium* (GTC 00603), *M. avium* subsp *paratuberculosis* (ATCC 19698), *M. avium* subsp. *hominissuis* (ATCC 19978) and *M. avium* subsp *silvaticum* (ATCC 49884) were used as standard strains. MAH isolates were recovered from (a) sputum of patients who were diagnosed with pMAC infection (National Hospital Organization, Higashinagoya National Hospital, *n*=69), according to the official guidelines of the American Thoracic Society/Infectious Diseases Society of America (Griffith *et al.*, 2007); (b) blood culture samples of HIV-seropositive patients with disseminated MAC infection (HIV-MAC) (*n*=28), provided by the National Center for Global Health and Medicine, formerly called the International Medical Center of Japan; and (c) intestinal lymph nodes of swine reared for edible meat (*n*=23), provided by the University of the Ryukyus, Japan.

Culture conditions and preparation of genomic DNA. MAC isolates were incubated at 37 °C for 1 to 3 weeks in 5 ml Middlebrook 7H9 liquid medium supplemented with 10 % oleic acid–albumin–dextrose–catalase enrichment. These cultures were used to inoculate 5 ml Mycobroth liquid medium (Kyokuto), which was then incubated at 37 °C until reaching an absorbance of 0.2 at 530 nm. DNA was then extracted from the cells using InstaGene Matrix (Bio-Rad Laboratories) according to the manufacturer's instructions. The purified DNA was stored at −20 °C until it was needed for use.

Table 1. The 16S−23S rDNA ITS sequence of described and new sequevars of *M. avium* subsp. *hominissuis*

Sequevar	Nucleotide sequences of the 16S–23S rDNA ITS																		GenBank accession numbers
	353	354	355	356	357	358	359	360	386	387	388	389	390	391	392	393	394	396	
Mav-A	C	T	T	C	G	T	G	G	T	C	T	T	T	T	T	T	–	A	L07855
Mav-B	C	T	T	G	G	T	G	G	T	C	T	T	T	T	T	T	–	A	L07856
Mav-C	C	T	T	C	G	T	G	G	T	C	C	T	T	T	T	T	–	A	L07857
Mav-D	C	T	T	C	G	T	G	G	T	C	G	T	T	T	T	T	–	A	L07858
Mav-E	C	T	T	T	A	T	G	G	T	C	T	T	T	T	T	T	T	A	Z46422
Mav-F	C	T	T	C	G	T	G	G	T	C	–	T	T	T	T	T	–	A	AF315838
Mav-G	C	T	T	T	G	T	G	G	T	C	T	T	T	T	T	T	–	A	AF315839
Mav-Q	C	T	T	C	A	T	G	C	T	C	–	T	T	T	T	T	–	A	LC176866
Mav-R	C	T	T	C	T	T	G	G	T	C	–	T	T	T	T	T	–	A	LC176867
Mav-S	C	T	T	C	A	T	G	G	T	C	–	T	T	T	T	T	–	A	LC176868

Insertion sequence analysis, *hsp65* single nucleotide polymorphism (SNP) analysis and detection of IS*Mav6*. The genomic DNA from MAH isolates was subjected to PCR analysis for detection of IS*1245*, IS*1311* and FR300 as described by Ichikawa *et al.* (2009). To determine subspecies of *M. avium* isolates, sequence analysis of the 3′ fragment of the *hsp65* gene was performed according to the methods described by Turenne *et al.* (2006). The nucleotide sequences of the *hsp65* gene thus obtained were compared with the reported data by BLAST analysis using the NCBI server (http://www.ncbi.nlm.nih.gov). Alignment was performed with CLC Sequence Viewer 4.6.2 (CLC Bio). The detection of IS*Mav6* was performed by PCR using specific primer sets designed for MAH, as previously described by Ichikawa *et al.* (2009) and Iwamoto *et al.* (2012). Ichikawa *et al.* (2009) previously reported that the standard strain *M. avium* 104 did not possess IS*Mav6*. So, it was used as negative control for MAH in the PCR analysis.

MATR-VNTR analysis. Primer sets for 13 MAH VNTR loci (MATR-VNTR; excluding MATR-VNTR-9, 14) were used in the VNTR analysis, as previously described by Ichikawa *et al.* (2010) and Inagaki *et al.* (2009). Minimum spanning tree (MST) analysis based on VNTR genotypes was performed using the Bionumerics software (version 7.0; Applied Maths N. V.) to reconstruct a hypothetical phylogenetic tree for the MAH isolates. The reconstruction of the phylogenetic tree was performed by first selecting a categorical coefficient. The priority rule was set so that the type that had the highest number of single-locus variants would be linked first. Creation of hypothetical types was not at liberty. The Manhattan distance matrix data of the allelic profiles of the VNTR loci were generated using the following formula: Σ [xn−yn], where xn and yn are the number of repeat units in the nth mini satellite locus of MAH isolates x and y, respectively.

16S–23S rDNA ITS sequence analysis. The clinical isolates were identified by molecular probe (Gen-Probe). Mycobacterial colonies were selected from Middlebrook medium plates (Becton Dickinson), grown in liquid Middlebrook medium and then typed by 16S–23S rDNA ITS nucleotide sequencing as described below. We performed 16S–23S rDNA ITS analysis according to Novi *et al.* (2000). Sequencing of the products was performed by the department of sequencing at the Hokkaido System Science. The nucleotide sequences of the 16S–23S rDNA ITS gene thus obtained were compared with the reported data by BLAST analysis using the NCBI server (http://www.ncbi.nlm.nih.gov).

Susceptibility of CLA. Susceptibility of CLA was determined using microdilution with BrothMIC NTM (Kyokuto Pharmaceutical Industrial) according to the manufacturer's instructions and in compliance with NCCLS/CLSI methods. CLA resistance was determined by MIC of CLA >32 mg ml^{-1}.

Statistical analysis. The genetic distances of MAC strains from three different origins, which were estimated from the Manhattan distance matrix data using *M. avium* 104 as a reference strain, were compared. Statistical analysis was performed using GraphPad Prism (version 6.0) for Mac (GraphPad Software). Statistical significance was taken as $P<0.05$.

RESULTS

hsp65 SNP analysis

Five types of *hsp65* sequevars were identified from 120 isolates using *hsp65* 3′-fragment PCR analysis to evaluate the presence of the discriminatory SNP. All 120 MAC clinical isolates were identified as MAH. The distribution of *hsp65* sequevars presented differences associated with the host of the isolates; isolates from swine were markedly different from human isolates (pMAC and HIV-MAC). Our data show the absence of code 2 and code 15 sequevars in swine strains, which predominate in strains from pMAC (code 15 occurrence: 39/69, 57 %) and HIV-MAC (code 15 occurrence: 13/28, 46 %). The code 2 and code 15 sequevars had a higher prevalence of IS*Mav6* (14/26, 54 %, and 33/52, 63 %, respectively) than code 1 sequevars (6/36, 17 %) (Table 2).

MATR-VNTR analysis

The genetic distances of the clinical isolates from three different origins were estimated by using a multiple comparison test and *M. avium* 104 as a clinically unbiased standard. Horizontal lines indicate mean values for the Manhattan distance from the standard strain *M. avium* 104. The genetic distance from the standard strain *M. avium* 104 in MAH isolates from HIV-MAC or swine was significantly closer than that in isolates from pMAC ($P<0.001$) (Fig. 1). We next compared the 13 loci VNTR genotypic profiles using MST-based phylogenetic analyses (Fig. 2). The cluster analysis showed that isolates from pMAC and swine grouped into distinct VNTR-type clusters, respectively. Isolates from blood culture in HIV-MAC were scattered throughout both clusters.

16S–23S rDNA ITS sequevars

PCR amplification 16S–23S rDNA ITS resulted in the detection of a single band of 480 bp in all the strains. Sequence analysis identified three major distinct *M. avium* sequevars, Mav-A, Mav-B and Mav-F (Frothingham & Wilson, 1993; Martha *et al.*, 2006; Mijs *et al.*, 2002), and three new sequevars, Mav-Q, R and S, in isolates from human patients. No isolate belonged to the previously reported Mav-C, Mav-D, Mav-E and Mav-G sequevars (Martha *et al.*, 2006). The majority of pMAC strains belonged to Mav-A (35/69, 51 %) and Mav-F (25/69, 36 %); on the other hand, in swine strains, Mav-B (15/23, 65 %) was predominant, and no Mav-F was found. These three ITS sequevars (Mav-A, Mav-B and Mav-F), were detected almost equally in the HIV-MAC strains (Fig. 3). With respect to the new sequevars, two were Mav-Q, one was Mav-R and three were Mav-S, all but one was recovered from pMAC strains, with the one remaining Mav-S isolated from blood culture of HIV-MAC patients (Table 3).

Table 2. The distribution of *hsp65* sequevars among the different hosts and prevalence of IS*Mav6*

	pMAC strains (*n*=69)	HIV positive (*n*=28)	Swine strains (*n*=23)	No. of IS*Mav6*
Code 1	4	12	20	6
Code 2	24	2	0	14
Code 3	2	1	0	1
Code 9	0	0	3	0
Code 15	39	13	0	33

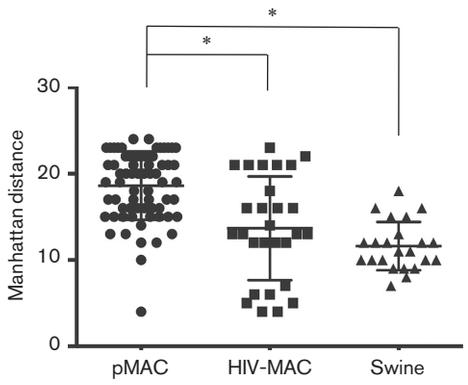

Fig. 1. The genetic distance of MAC isolates recovered from pMAC patients, HIV-MAC patients and swine calculated from the standard strain *M. avium* 104. The genetic distance, as assessed by Manhattan distance from *M. avium* 104, was significantly greater in *M. avium* isolates from 69 patients with pMAC disease compared with *M. avium* isolates from 28 HIV-positive patients and 23 swine ($P<0.0001$). Horizontal lines indicate mean values for the Manhattan distance from the standard strain *M. avium* 104. *$P<0.001$ was considered significant.

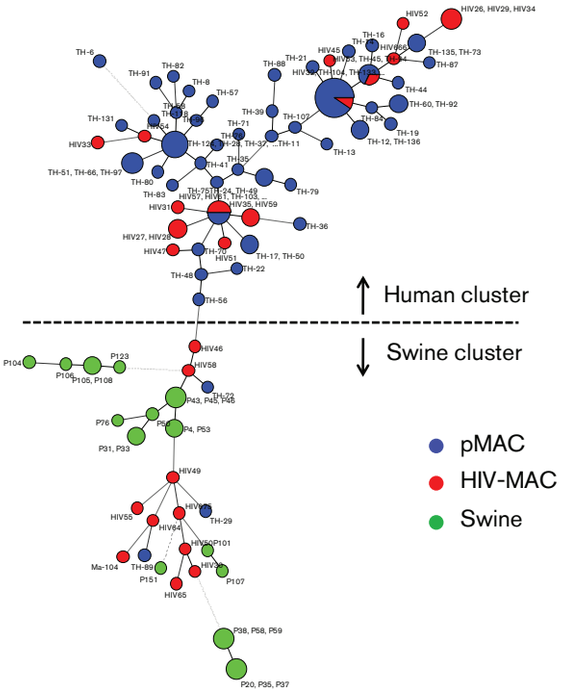

Fig. 2. An MST based on 13-VNTR genotyping for 120 MAH isolates from three different hosts. Circles correspond to the different VNTR genotypes. The circle sizes are proportional to the number of isolates sharing the identical pattern. Strains were isolated from 69 patients with pMAC (blue), 28 patients with HIV-MAC (red) and 23 swine. Heavy lines connecting two types denote single-locus variants, and thin lines connect double-locus variants.

Possession of IS*Mav6*

The PCR analysis revealed that IS*Mav6* was uniquely distributed among the MAH isolates. Specifically, IS*Mav6* was highly prevalent among human clinical isolates (61/97, 63 %). There were 48 strains that possessed IS*Mav6* among 69 pMAC strains (48/69, 70 %). These 48 strains comprised 22 strains (22/35, 63 %) in Mav-A, 2 strains (2/4, 50 %) in Mav-B and 20 strains (20/25, 80 %) in Mav-F. Almost new sequevars, Mav-Q, Mav-R and Mav-S, belonging to pMAC strains had IS*Mav6* (4/5, 80 %) (Table 3). In the case of the HIV-MAC strains, 33 % (3/9) of Mav-A, 20 % of (2/10) Mav-B and 87.5 % (7/8) of Mav-F isolates possessed IS*Mav6*. On the contrary, IS*Mav6* was not detected in swine strains. Human clinical isolates, in combination of pMAC with HIV-MAC, Mav-F, showed a greater prevalence of IS*Mav6* compared with Mav-A and Mav-B. Overall, Mav-B had a low prevalence of IS*Mav6*.

Susceptibility to CLA

Fifteen CLA-resistant strains were found from human clinical isolates, comprising 11 pMAC (11/69, 16 %) and 4 HIV-

Table 3. Distribution of IS*Mav6*-positive and CLA-resistant isolates among the different hosts in the 16S–23S rDNA ITS sequevars

	pMAC (n=69)			HIV-MAC (n=28)			Swine (n=23)		
	Total	IS*Mav6*	CLA resistance	Total	IS*Mav6*	CLA resistance	Total	IS*Mav6*	CLA resistance
Mav-A	35	22 (63 %)	5 (14 %)	9	3 (33 %)	0 (0 %)	8	0 (0 %)	0 (0 %)
Mav-B	4	2 (50 %)	0 (0 %)	10	2 (20 %)	0 (0 %)	15	0 (0 %)	0 (0 %)
Mav-F	25	20 (80 %)	5 (20 %)	8	7 (88 %)	3 (38 %)	0	0 (0 %)	0 (0 %)
Mav-Q	2	2 (100 %)	0 (0 %)	0	0 (0 %)	0 (0 %)	0	0 (0 %)	0 (0 %)
Mav-R	1	1 (100 %)	0 (0 %)	0	0 (0 %)	0 (0 %)	0	0 (0 %)	0 (0 %)
Mav-S	2	1 (50 %)	1 (50 %)	1	1 (100 %)	1 (100 %)	0	0 (0 %)	0 (0 %)
		48 (70 %)	11 (16 %)		13 (46 %)	4 (14 %)		0 (0 %)	0 (0 %)

CLA resistance: MIC >32 μg ml^{-1}.

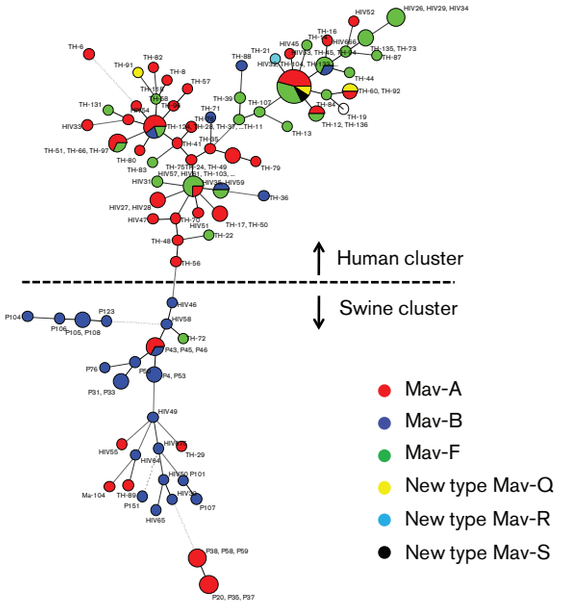

Fig. 3. The distribution of 16S–23S rDNA ITS sequevar on the MST based on VNTR genotyping. The ITS sequence data show that *M. avium* strains belonging to sequevar Mav-A and Mav-F were isolated from the majority of human infections. In the strains from swine, Mav-B was predominant, and no Mav-F was found. All three ITS sequevars, Mav-A, Mav-B and Mav-F, were detected almost equally in HIV-MAC strains.

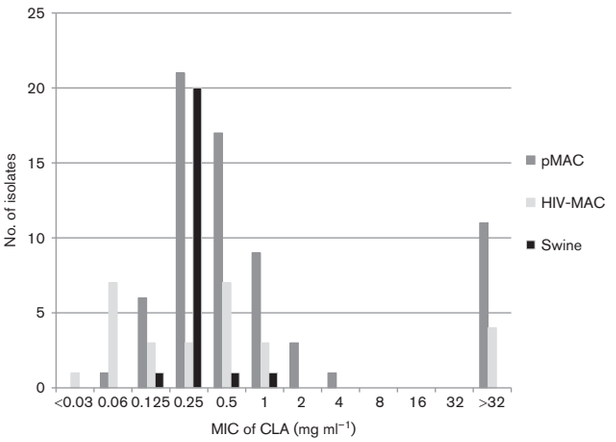

Fig. 4. Comparison of MIC of CLA for *M. avium* isolates from patients with pulmonary disease, HIV-positive patients and swine. Among *M. avium* isolates from pMAC patients and HIV-positive patients, there were some isolates highly resistant to CLA (MIC of CLA >32 mg ml^{-1}). All isolates from swine were susceptible to CLA.

MAC (4/11, 36 %) isolates. No CLA resistance was detected in swine isolates (Fig. 4). In 16S–23S rDNA ITS sequevar analysis, Mav-A and Mav-F strains had 10 % (5/52) and 24 % (8/33) CLA resistance, respectively. In contrast, all 29 Mav-B strains, including 14 human samples and 15 swine, had no CLA resistance (Table 3).

DISCUSSION

The present study showed genetic diversity of MAH strains recovered from human and swine, which was based on analyses of the *hsp65* sequevars, 16S–23S rDNA ITS sequevars, VNTR profiles, possession of IS*Mav6* and susceptibility to CLA.

First, our data revealed distinctive differences in genetic characteristics between pMAC strains isolated from respiratory samples of patients with pulmonary diseases and swine in Japan. Major *hsp65* sequence codes of the pMAC isolates from human were codes 2 and 15; on the other hand, code 1 was prominent in swine isolates. The pMAC isolates tended to form distinctive clusters from swine isolates in the MST based on 13-VNTR genotyping, which is consistent with the previous study by Iwamoto *et al.* (2012). Strikingly, blotting the 16S–23S rDNA ITS sequevars on the same MST based on the 13-VNTR genotyping indicated the similar separate pattern of distribution, Mav-A and Mav-F as major sequevars in human isolate cluster and Mav-B in swine isolate cluster. Three new sequevars, Mav-Q, Mav-R and Mav-S, which were found from six isolates, also belonged to human cluster. Together with the other characteristics of no possession of IS*Mav6* and no CLA resistance, these results suggested the distinctive genetic evolutionary lineage of pMAC strains and swine isolates.

Interestingly, Mav-F, one of the major 16S–23S rDNA ITS sequevars in human MAH isolates in Japan, appeared to be rare in the USA, which showed high prevalence rate of IS*Mav6* and relatively high CLA resistance rate (Iakhiaeva *et al.*, 2016). The predominant sequevar of human isolates in the USA was Mav-A and Mav-B, the latter of which is a major sequevar of swine isolates in Japan. Iakhiaeva *et al.* (2016) revealed the difference in allelic diversity in VNTR between pMAC isolates from USA and Japan. These findings may account for the genetic diversity of pMAC isolates from Japan and USA, which was compatible with our previous study (Ichikawa *et al.*, 2015). Further investigations were warranted for elucidation whether these differences were derived from only geographical diversity of isolation or genetic background related to pathogenesis and route of infection of MAH causing pulmonary or disseminated diseases.

Second, genetic feature of HIV-MAC strains was characteristic in terms of the distribution in the MST based on the 13-VNTR genotyping (Fig. 2) and possession of IS*Mav6*. They scattered in both human and swine clusters in the MST, and about 40 % of HIV-MAC isolates possessed IS*Mav6*, while the possession rates of pMAC and swine isolates were more than 70 % and 0 %, respectively. Several studies using the IS*1245*-based restriction fragment length

polymorphism analysis has demonstrated that AIDS patients are infected by unrelated, highly variable strains (Bono *et al.*, 1995; Guerrero *et al.*, 1995; Lari *et al.*, 1998; Ritacco *et al.*, 1998). Analyses with MST based on the 13-VNTR genotyping and Manhattan distance from *M. avium* 104 (Fig. 1) in this study showed wide diversity of HIV-MAC isolates, which was in accordance with those previous studies. As Fig. 2 showed, no less than 10 cases (36 %) of 28 HIV-MAC cases were included in swine cluster. This result suggested that strains which cause swine diseases might be likely to cause disease in immunocompromised subjects such as HIV patients, and this proportion was considered as significant. Most of the isolates from HIV-positive patients which belonged to swine cluster were typed as sequevar Mav-B according to the 16S–23S rDNA ITS sequence analysis. The predominance of strains of sequevar Mav-B in disseminated infections in AIDS patients has been reported previously (De Smet *et al.*, 1995; Frothingham *et al.*, 1994; Novi *et al.*, 2000), which was compatible with our study, suggesting the common infectious aetiology.

Third, CLA resistance and possession of IS*Mav6* were characteristic in human isolates of MAH. Highly CLA-resistant isolates which showed MIC more than 32 mg ml^{-1} were found in 16 % of pMAC strains, 22 % of HIV-MAC strains and 0 % in swine strains, respectively. This CLA resistance of MAH appeared to be developed on exposure to CLA which might be used against pulmonary or disseminated MAC diseases or other infectious diseases or used as an immunomodulatory agent (Griffith *et al.*, 2007). No Mav-B strains had any CLA resistance. IS*Mav6* were detected in only human MAH isolates especially in pMAC isolates. This insertion sequence was found to be inserted at several sites on the chromosome of MAH strains (Ichikawa *et al.*, 2009) and recently was reported to be related with the resistance to moxifloxacin (Kim *et al.*, 2016). These findings suggested that acquisition of IS*Mav6* is one of the adaptive processes for MAH strains to the environments where they reside or cause disease.

There are a few limitations in this study. First, the sample size may not be sufficient to detect significant differences between the genetic characteristics of the clusters of MAH isolates, particularly for isolates from HIV-MAC and swine. Second, all the isolates from humans and swine were collected only in Japan, although from diverse geographical area. Therefore, further epidemiological study with more isolates and more participant countries is warranted to confirm the differences in genetic backgrounds of the MAH isolates.

In conclusion, molecular typing and genetic characterization of MAH isolates from diverse hosts gave insights to increase our understanding of the epidemiology of infectious diseases caused by MAH. These molecular typing techniques such as VNTR or 16S–23S rDNA ITS sequence analysis may be useful to classify MAH strains to study the epidemiology and pathogenesis of disease caused by these isolates in normal versus immunocompromised subjects. Further search for determinants involved in pathogenicity and development of the relevant molecular genotyping assay will contribute to the clarification of many aspects of MAH infections, leading to more effective measures for the treatment of infectious diseases caused by MAH.

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