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Bacteriology

Genotyping of swine *Mycobacterium avium* subsp. *hominissuis* isolates from Kyushu, Japan

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ABSTRACT. The incidence of diseases caused by nontuberculous mycobacteria (NTM) is increasing annually worldwide, including Japan. *Mycobacterium avium* subsp. *hominissuis* (MAH) is one of the most common NTM species responsible for chronic lung diseases in animals and humans. In the current study, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing was employed to characterize the genetic diversity of swine MAH isolates from Kyushu, Japan. In total, 309 isolates were obtained from the lymph nodes of 107 pigs not displaying any clinical signs of disease, of which 307 were identified as MAH, comprising 173 strains. Based on eight established MIRU-VNTR loci, the MAH strains represented 50 genotypes constituting three lineages, and 29 had not been described in the Mac French National Institute for Agricultural Research Nouzilly MIRU-VNTR (Mac-INMV) database. MAH was the dominant *M. avium* complex (MAC) in pigs from Kyushu, and there was high genetic diversity among genotype profiles of MAH from Kyushu. We identified three predominant genotype profiles in the tested area sharing high relatedness with genotype profiles of strains isolated in European countries. MAH was the most common NTM in pigs from Kyushu and exhibited high diversity, with new strain-derived genotypes.

KEY WORDS: genotyping, Kyushu, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR), *Mycobacterium avium* subsp. *hominissuis*, nontuberculous mycobacteria

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Mycobacterium avium subsp. *hominissuis* (MAH), belonging to the *M. avium* complex (MAC), is the most frequently encountered nontuberculous mycobacteria (NTM). MAH strains are maintained or persist in humans, other animals, and the environment. In humans, NTM cause pulmonary nontuberculous mycobacterial disease (PNTMD) [19, 27], the incidence of which is increasing worldwide, including Japan [17, 19]. Pigs are the animals most susceptible to MAC infection, especially MAH [1, 10, 16, 18]. Pig breeding environments (bedding materials, water, and facilities used in pig husbandry) are a well-known source of MAH infection [16, 21]. In fact, MAH strains isolated from humans were found to be genetically similar to those in pigs in Japan [15].

MAH infection in pigs is characterized by granulomatous lesions in the lymph nodes of the digestive system [3]. Since most pigs do not show any clinical signs of infection, visceral organs, including lesions, are excluded from processing by macroscopic inspection at the slaughterhouse. An increase in the number of affected pigs will result in severe economic losses. Another problem associated with meat inspection is the inability to identify etiological agents other than by examining for macroscopic lesions. Furthermore, it is also quite difficult to determine the route of pathogen invasion in a pig farm, and the mode of pathogen propagation between individual animals.

Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) is a useful technique for characterizing the genetic diversity of isolates of MAC [4, 20], *M. avium* subsp. *paratuberculosis* [8, 31], and MAH [24–26] isolated from humans, other animals, and the environment. Eight MIRU-VNTR loci (292, X3, 25, 47, 3, 7, 10, and 32) can

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potentially be used for high-throughput, reproducible, effective discriminatory analysis of MAC [8, 14, 22, 28]. Using this approach, French National Institute for Agricultural Research (INRA) Nouzilly MIRU-VNTR (INMV) profile numbers are assigned based on information in the Mac-INMV database collated from many reports on mycobacterial genotyping by MIRU-VNTR to type *M. avium* subsp. *paratuberculosis*, MAH, *M. avium* subsp. *avium*, and *M. avium* subsp. *sylvaticum* (http//mac-inmv.tours.inra.fr). Although there have been some reports about relationship between animals- and human-derived MAH isolates based on 15 or more loci MIRU-VNTR, eight loci MIRU-VNTR was used to analyze the genetic comparison pig derived MAH in European countries [24, 25].

Kyushu, a southern island of Japan, hosts the biggest pig population in this country, and approximately a quarter of domestic pork meat is produced in this area (Ministry of Agriculture and Forestry and Fisheries, 2018). Although some outbreaks of MAC in pig farms have been reported in Japan, including in Kyushu [9], detailed analysis of the isolated bacteria, such as genotyping, has not been performed. The purpose of the current study was to elucidate the genetic diversity of MAH isolated from pigs in Kyushu, Japan.

MATERIALS AND METHODS

Sampling of pig lymph nodes

A total of 107 mesenteric lymph nodes with granuloma lesions from pigs aged ~6 months old and suspected of carrying Mycobacterium infections were used in this study. The samples from pigs raised on 15 farms (farms A–O) in Kyushu were obtained during 2012–2016. The farms selected for the survey operated independently, with no relationships between them. No animals exhibited any clinical signs of infection before delivery to the slaughterhouse, and inflammation of mesenteric lymph nodes (nodular lymphadenitis) was first observed by visceral inspection by veterinarians after slaughter. When lesions were found in many animals from a given farm, three tissue samples were randomly selected and subjected to microbial examination. When lesions were identified in many animals, every time pigs from a given farm were brought to the slaughterhouse, animals from that farm were sampled several times. Lymph node portions (0.2–0.5 g) were preserved at −20°C for bacterial culture, isolation, and identification. Bacteria were cultured on Middlebrook 7H10 agar (BD Difco, Sparks, MD, U.S.A.) and the acid-fastness of colonies was confirmed by Ziehl-Neelsen staining as described elsewhere [5]. Information on isolation year, farm, and pig source were recorded to trace strain information.

Genetic identification

DNA was extracted from single bacterial colonies grown on Middlebrook 7H10 agar by boiling as described elsewhere [11]. Multiplex PCR followed by IS901 flanking region (FR) 300 PCR were performed for species- and subspecies-level identification. Multiplex PCR involved denaturation at 95°C for 5 min, followed by 25 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min. Following multiplex PCR, IS901 FR300 PCR analysis was performed for subspecies-level identification. Thermal cycling involved denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 7 min. For multiplex PCR, MYCOGEN-F and MYCOGEN-R PCR primers were used, designed to specifically amplify only a single 1,030 bp product indicative of *Mycobacterium* spp. [6]. Meanwhile, MYCOGEN-F and MYCAV-R primers were designed to specifically amplify both a 1,030 bp product and a 180 bp DNA fragment from *M. avium* [6]. MYCINT-F and MYCOGEN-R primers were designed to specifically amplify an 850 bp DNA fragment from *M. intracelullare* [6]. For IS901 FR300 PCR forward and reverse primers, an amplicon ~300 bp in size indicated MAH isolates [23].

Genotyping of MAH isolates

For genotyping analysis, loci 292, X3, 25, 47, 3, 7, 10, and 32 were employed. For all loci other than loci 3 and 10, PCR involved an initial incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and a final incubation at 72°C for 7 min. For loci 3 and 10, an initial denaturation was performed at 95°C for 19 min, followed by 40 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final incubation at 72°C for 7 min. Primers used in this analysis were as described elsewhere [13, 14].

Allelic diversity (h) was calculated by the equation h=1-Σx_i^2[N/(N-1)], as described elsewhere [29], where x_i is the frequency of the *i-th* allele at the loci, N is the total number of isolates, and N/(N-1) is the correction factor. The discriminating power of MIRU-VNTR was calculated using the Hunter-Guston discriminatory index (HGDI) as described elsewhere [12]. HGDI was calculated by the following equation:

$$HGDI = 1 - \frac{1}{[N(N-1)]} \sum_{j=1}^{x} [n_j(n_j-1)]$$

where n_j is the number of isolates in the *i*-th cluster. A dendrogram of VNTR data was then generated using R software ver. 3.5.1 (https://www.r-project.org/). The matrix distance data profile was calculated using the *Euclidean* method, and genotype profiles were compared using the neighbor-joining clustering algorithm available in the phylogenetic and evolution (ape) package. The Mac-INMV database (http:/mac-inmv.tours.inra.fr/) version 2.1 was used as a reference and for comparison of genotype profiles. The lineage grouping of genotype profiles in samples, and clustering in samples and reference genotype profiles, was performed according to genetic distance based on genotype profile data.

Genetic relatedness between Kyushu strains and reference strains were estimated by minimum spanning tree (MST) to create a map displaying the relative positions of genotype profiles, based on genetic distance. Genetic distances were calculated by *Euclidean* methods using the following formula with eight loci MIRU-VNTR data as described elsewhere [15]:

$$D_{pq} = \sqrt{\sum_{k=1}^{8}\sum_{i=1}^{n}(P_{ki} - Q_{ki})^2}$$

where k represents the number of locus were used (a total of eight loci in this study), n is the highest copy number of tandem repeat units, and P_{ki} and Q_{ki} is the proportion of strains with the i-th number of tandem repeat units at locus k of population P and Q. MST analysis in this study was performed by the *igraph* packages function in R software version 3.5.1 (https://www.r-project.org/).

Strain determination

Isolates yielding the same genotype profile by MIRU-VNTR from a single pig were counted as one strain, whereas isolates yielding different genotype profiles by MIRU-VNTR from a single pig were counted as different strains.

Statistical analysis

χ^2 analysis was used to determine differences between groups by population and character strain comparisons. χ^2 analysis was performed using the MASS statistical tool in the R software (https://www.r-project.org/). The threshold for significant differences was set as $P<0.05$.

RESULTS

From 107 mesenteric lymph nodes, 309 colonies were isolated on Middlebrook 7H10 agar. Based on multiplex PCR and IS901 FR300 PCR, 2/309 isolates were identified as *Mycobacterium* spp. and 307/309 were identified as MAH. A total of 173 MAH strains among the 307 isolates were finally verified as independent strains and used for genotyping, of which 145 strains clustered in 22 genotype profiles and 28 strains were represented as single profiles. Based on MIRU-VNTR, the Kyushu strains yielded 50 distinct genotype profiles (ky01–ky50) and belonged to three lineages. PCR products for locus 32 were not amplified in 16 strains, distributed in 14 genotype profiles (Fig. 1).

Genotyping analysis of 17 genotype profiles matched with profiles in the Mac-INMV database, and two genotype profiles (ky26 and ky29) shared high genetic similarity (identity for seven MIRU-VNTR loci and no PCR product for locus 32) with reference genotype profiles (INMV51 and INMV156). Furthermore, 29 genotype profiles did not match any genotype profiles in the reference strains (they were represented as new INMV patterns).

The dendrogram showed that Kyushu strains were distributed in three lineages: 122/173 strains (70.52%) represented lineage 1, 49/173 strains (28.32%) represented lineage 2, and 2/173 strains (1.16%) represented lineage 3. Lineage 3 contained two unique genotype profiles associated with a high copy number for locus 10.

Three genotype profiles with significantly higher strain populations than others ($P<0.05$) were represented in genotyping analysis of Kyushu strains. These three predominant genotype profiles were ky07 (22331118; 32/173), ky08 (22431118; 30/173), and ky33 (24231128; 16/173). The ky07 and ky08 profiles represent lineage 1, and the ky33 profile represents lineage 2 (Fig. 1).

In this study, we analyzed the genetic relatedness between Kyushu strains and references data of MAH in Japan [15] and Europe [25, 30] using phylogenetic analysis based on seven loci MIRU-VNTR (292, X3, 25, 47, 7, 10 and 32). Kyushu strains closely localized in not only pig isolates but also human isolates of Japan and Europe in MST analysis (Fig. 2). The genotype profiles of Kyusyu strains, ky26 and ky34, which were not the predominant strains, matched with those of human isolates in Japan. Fifteen genotype profiles of Kyushu strains, including the predominant strain ky08, matched with those pig isolates in other parts of Japan [15]. However, the genotype profiles of the predominant strains ky07 and ky33 were independent from those of pig isolates in Japan (Fig. 2).

The allelic diversity data (Table 1) revealed that locus 25 displayed the highest allelic diversity, with high diversity for locus 25 (h=0.668), followed by locus X3 (h=0.539) and locus 10 (h=0.520). Loci 3 and 7 did not exhibit allelic diversity (h=0.000), and locus 292 displayed low allelic diversity (h=0.300). Single locus analysis by MIRU-VNTR resulted in poor discrimination of the genetic diversity of MAH in Kyushu. Loci 3 and 7 were monomorphic. The best single locus discriminatory values were locus 25 (HGDI=0.695) followed by locus 10 (HGDI=0.530). Other loci resulted in low and moderate discrimination. Eight combinations of loci successfully discriminated MAH among Kyushu isolates (HGDI=0.921).

DISCUSSION

Genetic diversity of MAH in Kyushu based on eight locus MIRU-VNTR revealed a high level of diversity. Multi-locus analysis of MIRU-VNTR identified 50 different genotype profiles among Kyushu isolates. High genetic diversity of MAH in Japan was also reported for human and pig isolates [14, 15]. The distribution of genotype profiles in Kyushu was not correlated with geographic differences.

The current study revealed low similarity between MAH strains from different farms in Kyushu. These data indicate a low possibility of cross-contamination between farms in this region. The distribution of strains in Kyushu may be associated with the style

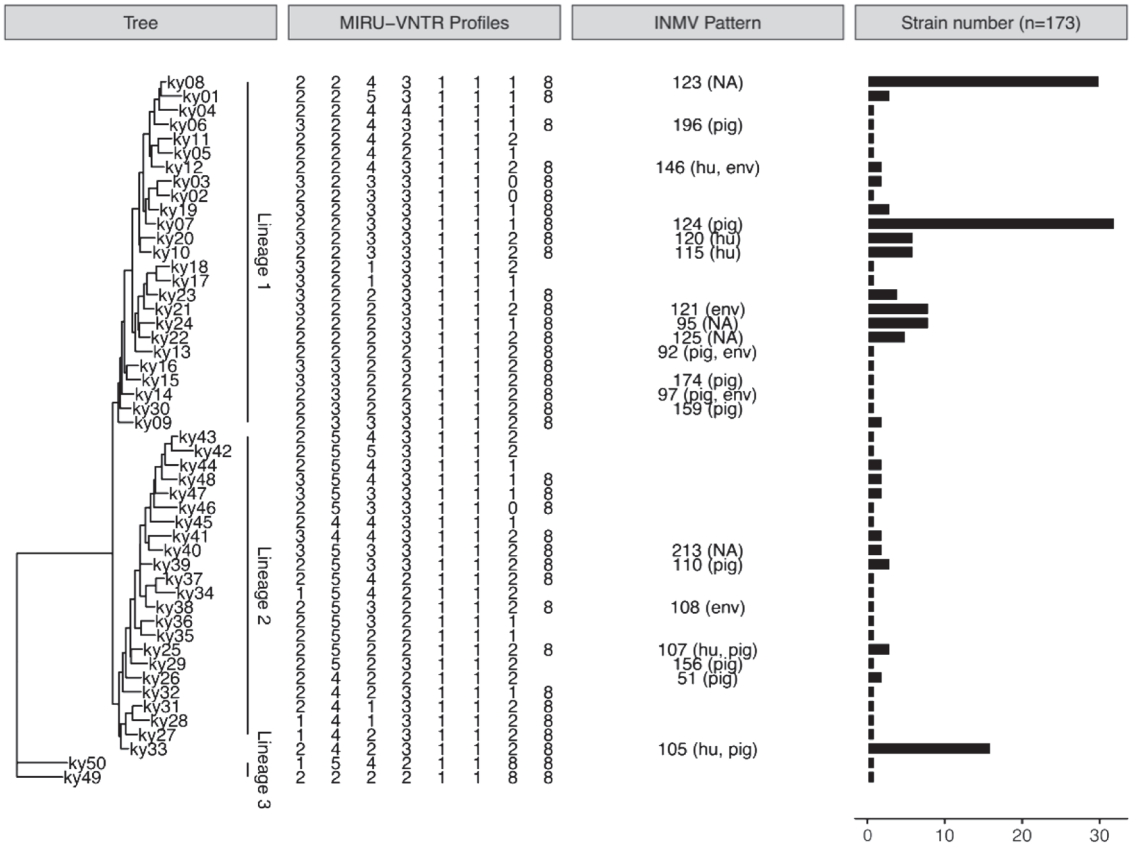

Fig. 1. Genetic characterization of *Mycobacterium avium* subsp. *hominissuis* strains isolated from pigs in Kyushu, Japan, based on eight locus Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) from samples collected between 2012–2016. The Mac-INMV number is specified in the INMV pattern panel. The dendrogram was constructed based on a genetic distance matrix and compared using the neighbor-joining clustering algorithm. hu, human isolates; pig, pig isolates; env, environmental isolates; NA, no PCR product.

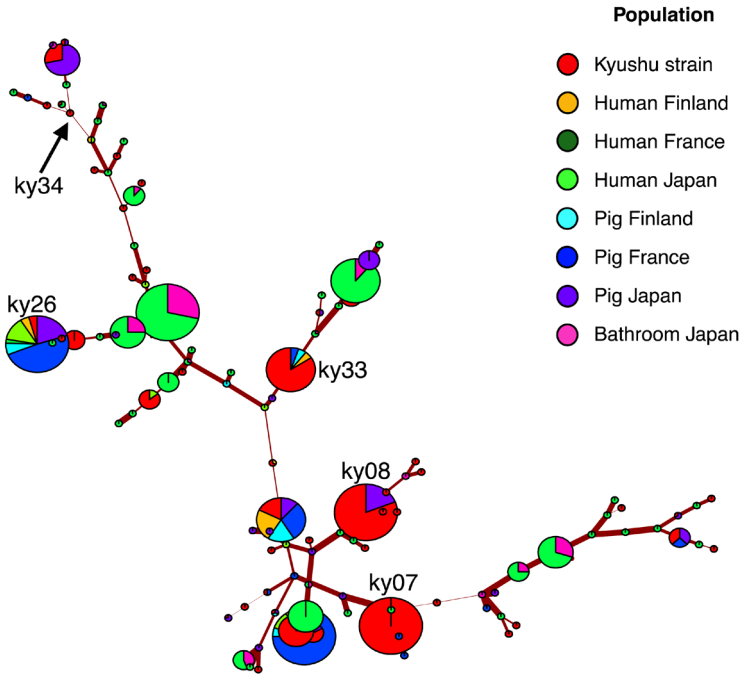

Fig. 2. Minimun spanning tree (MST) analysis and phylogenetic network of *Mycobacterium avium* subsp. *hominissuis* strains isolated from pigs in Kyusyu, Japan. MST analysis of Kyushu strains with Japan and Europe genotype profiles from humans, pigs and environments, based on seven locus (292, X3, 25, 47, 7, 10 and 32) MIR-VNTR. The circle sizes are proportional to the isolates sharing and identical genotype profiles number. Three type of edge line connecting denote single, double or multiple locus variant.

Table 1. Allelic frequency of the variable locus number and allelic diversity of swine *Mycobacterium avium* subsp. *hominissuis* isolated from Kyushu, Japan, in the years 2012–2016

Locus	0	1	2	3	4	5	8	Allelic diversity (*h*)[a)]
292		0.013	0.818	0.169				0.3
X3			0.632	0.036	0.212	0.121		0.539
25		0.013	0.375	0.371	0.228	0.013		0.668
47			0.107	0.889	0.003			0.195
3		1						0
7		1						0
10	0.016	0.498	0.479				0.007	0.52
32[b)]							1	0

a) Definitions: $0<h<0.3$, poor discrimination; $0.3<h<0.6$, moderate discrimination; $0.6<h<1$, high discrimination, as described by Sola *et al*. [29]. b) PCR product of loci 32 only showed in 155 strains and 18 strains no PCR product.

of pig farming, since pigs are intensively farmed in a single stay system, and never moved elsewhere because they were slaughtered at the same place that they were born. This multi-age farming system may contribute to horizontal infection and steady maintenance of MAH on pig farms. We found some continuous MAH infections in Kyushu; genotype profile ky07 was isolated in 2014 and 2015 from farm B, and genotype profile ky33 was isolated in 2015 and 2016 from farm K (data not shown). Pig barn environments probably contain most of the factors that result in repeated autoinfection of pigs with MAH. Sawdust, peat, or other kinds of bedding are reportedly one of the main sources of infections that result in repeated autoinfection of pigs with MAH [2]. Reusing sawdust for bedding in pig farms is common practice in Kyushu, and this may be one of the factors responsible for horizontal transmission. The failure of cleaning systems to eliminate MAH from pig farms leads to continuous infection over several farming periods.

Allelic diversity data (Table 1) showed that Kyushu strains shared similar characteristics with MAH isolated in other parts of Japan [14, 15]. When we compared seven locus MIRU-VNTR profiles between pig isolates from Kyushu with human isolates from other areas of Japan [14, 15], we revealed low genetic relatedness. Moreover, only two genotype profiles, ky16 and ky26, matched human *M. avium* isolated from patients with pulmonary granuloma in Nagoya between 2004 to 2008 [14].

Most genotype profiles of Kyushu strains revealed high relatedness with genotype profiles of Japan and Europe strains. These data suggested that high genetic diversity was revealed for MAH genotype profiles in pigs of Kyushu. Interestingly, two profiles, ky26 and ky34, matched with human isolates in Japan [15] (Fig. 2), however, pathogenic relevance of these isolates were unknown.

The three predominant genotype profiles of Kyushu strains (ky07, ky08, and ky33) were isolated from healthy pigs in Kyushu (Fig. 1). MAH with these genotype profiles have been registered in the Mac-INMV database as INMV124, INMV123, and INMV105, respectively and are believed to be related to pathogenicity. Ky07 profiles have been isolated from swine lymph nodes, genital swabs, aborted fetuses, uterus, sperm, and umbilical cord from a breeding sow in Germany [7] but not isolated in Japan. Ky08 profile were already reported from pig in Japan [15]. Ky33 profile has been isolated in European countries, but not isolated in Japan. This profile has been isolated from pig, and cattle in France [25], and pig and chicken in Finland [3]. These inconsistent results may be due to the age of pigs. We isolated MAH with these genotype profiles from growing-finishing pigs (around 6-months-old), while other groups isolated these MAH strains from sows. The infection period of MAH in pigs may correlate with the development of clinical signs. Further work to confirm the pathogenicity of the predominant MAH strains in Kyushu is in progress in our laboratory.

Although our study based on eight loci MIRU-VNTR revealed some genotype profiles of Kyushu strains were similar with those of human isolates [3, 25], 15 or more loci MIRU-VNTR analysis were used to analyze the relationship between animals- and human-derived MAH isolates. [24, 25]. Further analysis is needed to clarify the relationship between Kyusyu strains and human strains.

In conclusion, high genetic diversity was revealed for MAH genotype profiles in Kyushu, and the characteristics of genotype profiles were similar to those of MAH strains isolated in other parts of Japan. Three predominant strains were identified, all of which had been reported to not only infect pigs, but also humans, not only in Japan but also in European countries.

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