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Research paper

Molecular analysis and MIRU-VNTR typing of *Mycobacterium avium* subsp. *avium*, '*hominissuis*' and *silvaticum* strains of veterinary origin

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ABSTRACT

Besides *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *silvaticum* (MAS), and '*M. avium* subsp. *hominissuis*' (MAH) are equally important members of *M. avium* complex, with worldwide distribution and zoonotic potential. Genotypic discrimination is a prerequisite to epidemiological studies which can facilitate disease prevention through revealing infection sources and transmission routes. The primary aim of this study was to identify the genetic diversity within 135 MAA, 62 MAS, and 84 MAH strains isolated from wild and domestic mammals, reptiles and birds.

Strains were tested for the presence of large sequence polymorphism LSP^{A}17 and were submitted to Mycobacterial interspersed repetitive units-variable-number tandem repeat (MIRU-VNTR) analysis at 8 loci, including MIRU1, 2, 3, and 4, VNTR25, 32, and 259, and MATR9. In 12 strains *hsp*65 sequence code type was also determined.

LSP^{A}17 was present only in 19.9% of the strains. All LSP^{A}17 positive strains belonged to subspecies MAH. The discriminatory power of the MIRU-VNTR loci set used reached 0.9228. Altogether 54 different genotypes were detected. Within MAH, MAA, and MAS strains 33, 16, and 5 different genotypes were observed. The described genotypes were not restricted to geographic regions or host species, but proved to be subspecies specific.

Our knowledge about MAS is limited due to isolation and identification difficulties. This is the first study including a large number of MAS field strains. Our results demonstrate the high diversity of MAH and MAA strains and the relative uniformity of MAS strains.

1. Introduction

Mycobacterium avium subspecies are phenotypically diverse organisms with specific pathogenicity and host range characteristics (Rindi and Garzelli, 2014). The official classification based on numerical taxonomy was established by Thorel et al. (1990). *M. avium* subspecies *avium* (MAA) is the infectious agent of avian tuberculosis (Dhama et al., 2011). For the human/porcine type strains the designation '*M. avium* subspecies *hominissuis*' (MAH) was proposed by Mijs et al. (2002). *M. avium* subspecies *silvaticum* (MAS) has similarities to *M. avium* subspecies *paratuberculosis* (MAP) in its growth characteristics and is almost identical to MAA in its genomic sequence (Paustian et al., 2008). Due to the difficulties in the isolation and identification of the strains our knowledge about MAS is limited.

Infections caused by *M. avium* subspecies induce economic losses and public health problems. Improving control efforts and targeting transmission pathways require adequate understanding of the diversity and the distribution of *M. avium* subspecies within herds, host species, and geographic regions (Ahlstrom et al., 2015).

Large sequence polymorphisms (LSPs) are molecular markers of genetic diversity both in *M. tuberculosis* complex (Mostowy et al., 2002) and *M. avium* subspecies (Semret et al., 2004). According to Semret et al. (2004) genomic reduction through the loss of LSPs displays not only phylogenetic differences but also host species specificities and pathogenic properties.

Sequencing of *hsp*65 gene proved to be a useful tool to distinguish among subsets of the *M. avium* complex (Turenne et al., 2006). In their subsequent study Semret et al. (2006) associated *hps*65 gene sequence variants with LSP differences and suggested that the absence of LSP^{A}17 might be a feature of *hsp*65 code3 sequevar strains.

The Mycobacterial interspersed repetitive units-variable-number tandem repeat analysis (MIRU-VNTR) technique was described for typing and the assessment of genetic diversity among *M. avium* strains by Thibault et al. (2007). Since then several loci have been identified and different loci collections have been tested in order to reach a higher index of discrimination.

The primary aim of our study was to investigate the molecular diversity of MAA, MAH, and numerous MAS strains from veterinary

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origin by LSP^{A}17 analysis and a panel of MIRU-VNTR loci not tested earlier on MAS and MAH strains. Additionally, we were aiming to examine correlations between the detected genotypes and their epidemiological data.

2. Materials and methods

2.1. Mycobacteria strains, DNA extraction

The 281 strains included in the present study (Table 1) have been isolated between 2006 and 2015 and preserved in the strain collection of the Bacteriology Laboratory of Veterinary Diagnostic Directorate of the National Food Chain Safety Office (NFCSO) in Budapest, Hungary. Strains were grown in pure culture on Loewenstein-Jensen, and Middlebrook 7H11 (with MycobactinJ (Synbiotics Europe, Lyon, France)) slants at 37 °C.

DNA extraction was performed by QIAmp DNA Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

Wilton and Cousins (1992) was applied for identification of *M. avium* strains, while subspecies were distinguished by insertion sequence (IS) specific PCRs. IS*900* was examined according to Castellanos et al. (2009) and IS*901* and IS*1245* by Álvarez et al. (2008) method. Identification of MAS strains was performed using Rónai et al.'s (2015b). In the molecular identification tests MAA NCTC13034^T, MAS ATCC49884^T, and MAP ATCC19851 were used as amplification controls.

2.2. Molecular characterization and phylogenetic analysis

Large sequence polymorphism LSP^{A}17 was tested on 281 strains according to Semret et al. (2006). *Hsp*65 sequevar determination was performed on 12 strains by amplification and sequencing of the 3′ end of the *hsp*65 gene, according to Turenne et al. (2006).

MIRU-VNTR typing was performed on 281 strains by amplification of four MIRU (MIRU1, MIRU2, MIRU3, MIRU4), three VNTR (VNTR25, VNTR32, VNTR259), and one MATR (MATR9) loci described earlier by Bull et al. (2003), Castellanos et al. (2010), Millán et al. (2010) and Inagaki et al. (2009). PCR products were visualized on 2% agarose gel. The number of tandem repeats was determined from the size of the amplicons according to Bull et al. (2003) and Castellanos et al. (2010). In cases of small allelic differences PCR products were sequenced using an ABI Prism 3400 DNA Sequencer (Applied Biosystems).

The allelic diversity (h) of the different loci was calculated for the three subspecies combined and separately by the equation $h = n(1 - \Sigma x_i^2) / (n - 1)$, where n is the number of bacterial strains and x_i is the frequency of the ith allele at the locus (Kim et al., 2010). The Index of Discrimination (DI) was determined according to the formula described by Hunter and Gaston (1988). DI was calculated for each subspecies individually, for the strain collection as a whole, and for domestic and wild animals, and bird species separately. Epidemiologically related strains were excluded both from allelic diversity calculation and DI determination.

In order to determine the genetic relatedness of the different genotypes Neighbour-Joining (NJ) analysis was performed in MEGA6 (Tamura et al., 2013) along with MAP ATCC19851, MAA NCTC13034^T, and MAS ATCC49884^T reference strain (Fig. 1).

3. Results

The distribution of *M. avium* subspecies among different host groups (wild/domestic, mammals/birds) is presented in Table 1.

Large sequence polymorphism LSP^{A}17 was tested in all 281 strains. LSP^{A}17 was present only in 19.9% (56/281) of the strains, which all proved to be MAH. With the exception of one MAS strain, where no amplification occurred, all MAA and MAS strains lacked LSP^{A}17.

In 12 strains (1 MAA, 1 MAS, 4 LSP^{A}17 positive MAH, 6 LSP^{A}17 negative MAH) the 3′ end of the *hsp*65 gene was sequenced for sequevar code determination. MAA and MAS strains proved to be sequevar code4. Sequevars code1 and code3 were detected in both LSP^{A}17 positive and negative MAH strains.

The observed repeat numbers at the different loci and the calculated allelic diversities (h) for the three subspecies combined and separately are listed in Table 2. The highest allelic diversity was observed at locus

Table 1
M. avium subspecies distribution among different host groups in the present study.

Host species	Mycobacteria strains			
	MAA (n = 135)	MAH (n = 84)	MAS (n = 62)	Total (n = 281)
Domestic mammals	**58**	**54**	**5**	**117**
Cattle (*Bos taurus*)	14	40	5	59
Swine (*Sus scrofa domestica*)	44	14	0	58
Domestic birds	**10**	**0**	**0**	**10**
Chicken (*Gallus gallus domesticus*)	7	0	0	7
Duck (*Anas platyrhynchos domesticus*)	1	0	0	1
Pigeon (*Columba livia domestica*)	1	0	0	1
Turkey (*Meleagris gallopavo domesticus*)	1	0	0	1
Domestic animals	**68**	**54**	**5**	**127**
Wild mammals	**60**	**27**	**57**	**144**
Badger (*Meles meles*)	0	0	1	1
Mouflon (*Ovis orientalis*)	0	1	0	1
Red deer (*Cervus elaphus*)	2	15	10	27
Red fox (*Vulpes vulpes*)	8	0	2	10
Wild boar (*Sus scrofa*)	50	11	44	105
Wild birds	**6**	**2**	**0**	**8**
Long-eared owl (*Asio otus*)	1	0	0	1
Mallard (*Anas platyrhynchos*)	1	0	0	1
Peacock (*Pavo cristatus*)	0	1	0	1
Pochard (*Aythya ferina*)	1	0	0	1
Tauraco (*Tauraco sp.*)	1	1	0	2
Tragopan (*Tragopan sp.*)	1	0	0	1
Wigeon (*Anas penelope*)	1	0	0	1
Wild animals	**66**	**29**	**57**	**152**
Companion animals	**1**	**1**	**0**	**2**
Dog (*Canis lupus familiaris*)	0	1	0	1
Monitor lizard (*Varanus sp.*)	1	0	0	1

Abbreviations used: MAA: *Mycobacterium avium* subsp. *avium*, MAS: *Mycobacterium avium* subsp. *silvaticum*, MAH: '*Mycobacterium avium* subsp. *hominissuis*'.