Genetic variability of *Mycobacterium avium* subsp. *avium* of pig isolates

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ABSTRACT: The genetic diversity of 132 pig isolates of *Mycobacterium avium* subsp. *avium* (*MAA*) from the Czech Republic, the Slovak Republic and Slovenia was examined by IS*901* restriction fragment length polymorphism (RFLP) analysis with restriction endonuclease *Pvu*II. A total of 18 RFLP types were detected. The occurrence frequency of respective RFLP types varied between respective pig farms, with the exception of one RFLP type F found in 21 (34.4%) of 61 farms and in 10 (55.6%) of 18 farms in the Czech Republic and Slovak Republic, respectively. Two different RFLP types were detected in 5 (33.3%) of the 15 studied farms, from which more than one isolate were examined. These results show the low variability of the *MAA* isolates among the pig farms and the possibility of various sources of infection for pigs from infected farms.

Keywords: avian tuberculosis; food safety; molecular epidemiology; mycobacteriosis; zoonosis

Mycobacterium avium complex (*MAC*) infections on pig farms cause economic losses for the farmers. High losses result from the limitation or prohibition of the sale and movement of live animals from infected farms and from early culling of infected animals. In addition, meat and parenchymatous organs are classified as conditionally consumable if tuberculous lesions are found during the inspection of the slaughtered pigs (Komijn et al., 1999; Offermann et al., 1999; Pavlik et al., 2003). While, at present, the main causes of infection in pigs are *MAC* isolates of genotype IS*901*–, IS*1245*+ and serotypes 4 to 6, 8 to 11 and 21, designated as *M. a.* subsp. *hominissuis* (*MAH*; Mijs et al., 2002), *M. avium* subsp. *avium* (*MAA)* isolates of IS*901*+, IS*1245*+ genotype and serotypes 1 to 3 (Mijs et al., 2002; Bartos et al., 2006) should also be considered (Pavlik et al., 2000, 2003; Ocepek et al., 2003; Dvorska et al., 2004; Shitaye et al., 2006). Although *MAH* is commonly present in the environment, *MAA* is rarely isolated from the environment; the main source of this infection is birds (Pavlik et al., 2000).

A crucial aspect of control of the disease is the ability to determine where transmission is occurring in order to prevent further spread of infection. For the better understanding of *MAA* disease, different phenotyping and genotyping laboratory techniques have been applied. Serotyping (Wolinsky and Schaefer, 1973), the most frequently used phenotyping method, is limited by the prevalence of only three *MAA* serotypes and auto-agglutinating isolates. At present, genotyping (i.e. molecular-biological) methods are gradually replacing phenotyping methods. Several molecular methods were used for *MAA* genotyping: random amplified polymorphic DNA (RAPD; Pillai et al., 2001; Glawischnig et al., 2006), IS*1245*–IS*1311* spacer typing (Pate et al., 2005; Glawischnig et al., 2006)

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and mycobacterial interspersed repetitive units (MIRU) typing (Romano et al., 2005; Thibault et al., 2007). Although these methods are neither time consuming nor expensive, they have not been used routinely. Despite a number of disadvantages, the most commonly used methods in epidemiological studies of *MAA* infections are restriction fragment length polymorphism (RFLP; Ritacco et al., 1998; Dvorska et al., 2003, 2004, 2007) and pulsed-field gel electrophoresis (PFGE; Bono et al., 1995; Mobius et al., 2006).

RFLP analysis of *MAA* isolates takes advantage of the presence of the IS*901* insertion sequence, which is present in up to 14 copies in different localisations in the genome of different *MAA* isolates (Ritacco et al., 1998; Dvorska et al., 2003, 2004; Matlova et al., 2005). The number of copies ensures that resulting patterns are easy to read and they can be easily compared with the patterns of other isolates. Regardless, for the correct interpretation of the results of this method, a detailed study examining the genetic variability of isolates is necessary.

Based on the results of our previous studies on cattle and birds (Dvorska et al. 2004, 2007), we wanted to confirm these hypotheses: (i) pig isolates from different farms with no plausible epizootic link have a different pattern, (ii) isolates from the same pig farm have the same pattern and (iii) regional similarity among the patterns exists. For this purpose, we chose the pig isolates that originated from different farms without an epidemiological connection. On the farm, where *MAA* isolates from more pigs were available, these isolates were also included to describe the stability of the IS*901* RFLP pattern during the transmission or to reveal the possibility of more sources of infection for the pigs.

MATERIAL AND METHODS

M. a. avium isolates

A total of 132 pig isolates originating from collections of the Czech Republic (15 isolates from Veterinary Research Institute, Brno and 83 isolates from State Veterinary Institute, Prague), the Slovak Republic (21 isolates from State Veterinary Institute, Nitra) and Slovenia (13 isolates from Veterinary Faculty, University of Ljubljana). These isolates originated from slaughtered pigs from 82 farms and 42 different regions (from 15 farms, more than one isolate was examined from each); each isolate originated from a single animal from a known farm. All isolates were subcultured on solid media according to Stönebrink, Löwenstein-Jensen, Herrold (Herrold Egg Yolk medium) and on Middlebrook-7H11 medium.

PCR analysis

All isolates were tested for the presence of IS*901* and IS*1245* by the PCR method described previously (Bartos et al., 2006).

RFLP and data analysis

Mycobacterial DNA was isolated according to van Soolingen et al. (1998). Digestion with restriction endonuclease (RE), electrophoresis, blotting and hybridisation methods was performed according to Dvorska et al. (2003). DNA was digested with RE *Pvu*II. For the detection of IS*901*, the probe was prepared with the following primers: 5' -GCA ACG GTT GTT GCT TGA AA-3' and 5' -TGA TAC GGC CGG AAT CGC GT-3'. The length of the resulting amplicons was 1 108 bp (Kunze et al., 1992).

We used a categorical classification scheme to identify matched isolates: only the RFLP patterns with an identical number and position of IS*901* bands were considered to be a match. RFLP types were analysed by GelCompar software (Applied Maths, Version 4.1, Kortrijk, Belgium). Normalisation of the fingerprints was done using the molecular weight standard 1 kb DNA Ladder (ABGene, United Kingdom). The GelCompare software was used to calculate Dice coefficients of similarity and to cluster the isolates and generate dendrograms by the unweighted-pair group method (UPGMA) using linkage averages. The 260 optimisation and tolerance settings were both set at 2.0%. RFLP types were designed according to Dvorska et al. (2003). Four new RFLP types were designated as Z, AA, AB and AC.

RESULTS

Detected RFLP types

After typing of the 132 *MAA* isolates of genotype IS*901*+ and IS*1245*+ from 82 farms, 18 different RFLP types were obtained (Table 1): 14 RFLP

types have been described previously (Dvorska et al., 2003) and 4 RFLP types Z, AA, AB and AC were identified as new (Figure 1). All 18 distinct RFLP types were classified as a "multicopy pattern" and they were grouped into three clusters of > 69.7% similarity (Table 1, Figure 2). The most common RFLP types C, F, G and O representing 22.2% of detected RFLP types were present on 58 farms (70.7%). Seven unique RFLP types (38.9%) were present on only one farm (8.5%; Table 1).

The presence of RFLP types in different countries

After the examination of 98 *MAA* isolates from 61 farms situated in the Czech Republic, 16 various RFLP types were identified. Among these, three new IS*901* RFLP types were identified: Z, AB and AC (Figures 1 and 2). The most common RFLP type F was detected on 21 farms (Table 1). In the Slovak Republic, four various RFLP types of 21 *MAA* isolates, described

Table 1. Distribution of *Mycobacterium avium* subsp. *avium* isolates of different *Pvu*II RFLP types in three countries

*Pvu*II RFLP type[a]	*MAA* total No.	Czech Republic			Slovak Republic			Slovenia		
		MAA No.	farm		*MAA* No.	farm		*MAA* No.	farm	
			No.	designation[b]		No.	designation		No.	designation
C	28	25	10	15, 16, 18, 32, 33, 42, 45, 49, 51, 55	3	3	73, 74, 78	0	0	
D	4	4	3	25, 30, 33	0	0		0	0	
E	12	12	5	1, 35, 36, 40, 41	0	0		0	0	
F	33	23	21	2, 3, 6, 8, 11, 12, 14, 17, 22, 24, 27, 28, 31, 34, 44, 46, 47, 52, 54, 58, 60	10	10	62, 64, 67, 68, 69, 71, 72, 75, 76, 77	0	0	
G	10	5	5	9, 10, 31, 53, 61	5	3	63, 65, 79	0	0	
J	1	1	1	19	0	0		0	0	
K	4	1	1	48	3	2	66, 70	0	0	
L	5	5	1	39	0	0		0	0	
M	1	0	0		0	0		1	1	80
N	5	5	4	4, 13, 21, 56	0	0		0	0	
O	10	5	5	20, 23, 26, 43, 59	0	0		5	1	81
Q	4	4	4	5, 32, 37, 38	0	0		0	0	
T	1	1	1	50	0	0		0	0	
U	1	1	1	29	0	0		0	0	
Z	3	3	2	7, 57	0	0		0	0	
AA	7	0	0		0	0		7	1	82
AB	1	1	1	2	0	0		0	0	
AC	2	2	1	7	0	0		0	0	
Total	132	98	61	1–61	21	18	62–79	13	3	80–82

[a]designation of IS*901 Pvu*II RFLP types C to U according to Dvorska et al. (2003); new RFLP types Z, AA, AB and AC were described

[b]in five farms (designated and underlined 2, 7, 31, 32 and 33), two different RFLP types were detected

MAA = *Mycobacterium avium* subsp. *avium*

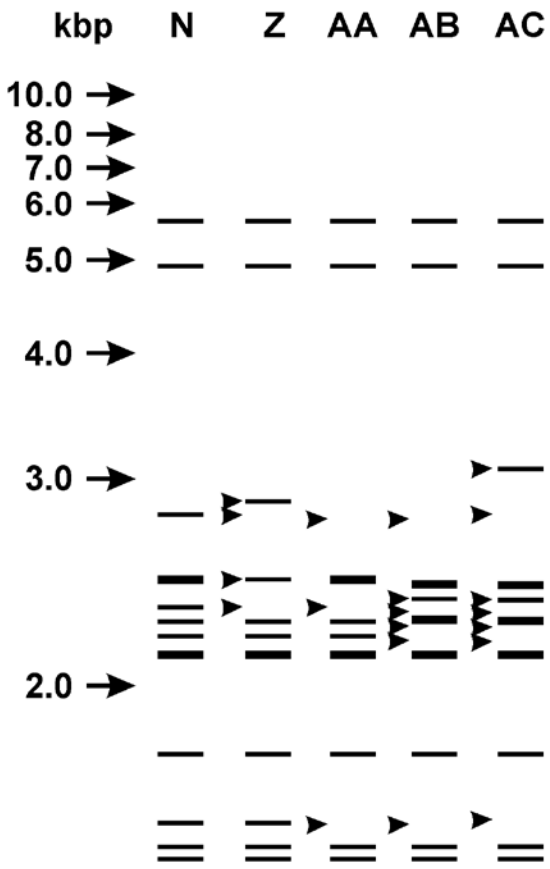

Figure 1. Four newly described IS*901* RFLP types Z, AA, AB and AC of the *M. a. avium* digestion with restriction endonuclease *Pvu*II and the previously described "reference" RFLP type N (Dvorska et al., 2003). A 1 kbp ladder (ABGene, United Kingdom) is situated on the left side of the diagram

also in the Czech Republic, were identified. The most common was also RFLP type F, which was detected on 10 farms (Table 1). In Slovenia, one new RFLP type AA and two different RFLP types were identified on three farms (Table 1, Figures 1 and 2). Dice coefficient similarity among isolates in each country or among the countries were > 70%.

The diversity of RFLP types on pig farms

The genetic diversity of pig isolates on each farm with more than one isolate (15 farms) was also investigated in the Czech Republic. Two different RFLP types only occurred at the same time on five (33.3%) farms (Table 1), the patterns demonstrated from one to five fragment differences and their Dice coefficient similarity were ranging between > 90% on farm No. 31 and > 70% on farms No. 7 and 32.

DISCUSSION

Although *MAA* is the causal agent of avian tuberculosis and rarely causes the infection in other animals e.g. pigs, there are a lot of gaps in the knowledge of this disease. These gaps are caused by the incorrect identification because some authors do not differentiate between *MAA* and *MAH* infection and due to a lack of appropriate typing methods. In spite of that, few epidemiological studies of *MAA* exist in which RFLP typing proved to be much more discriminatory than other molecular methods with the exception of PFGE (Ritacco et al., 1998; Dvorska et al., 2003, 2004, 2007; Pavlik et al., 2005). Our work was focused on studying the genetic variability of *MAA* isolates from pigs in three Central European countries by IS*901* RFLP analysis using one RE *Pvu*II. The isolates originated from either epidemiologically unrelated farms or groups from one farm (Table 1). Based on the results of our previous studies (Dvorska et al. 2004, 2007) the three hypotheses listed were:

(i) pig isolates from different farms with no plausible epizootic link have a different pattern,
(ii) isolates from the same pig farm have the same pattern,
(iii) regional similarity among the patterns exists.

The most common RFLP types C, F, G and O representing 22.2% of the detected RFLP types were present on 58 farms (70.7%). This indicates the low diversity of the isolates among the farms and that transmission between "unrelated" pig farms could appear. This is in disagreement with our previous hypothesis that pig isolates from different farms with no plausible epizootic link have a different pattern (Table 1). Due to a lack of information about the purchase of the animals and feed from the same farm or company, it is likely that pigs become infected from the other animals, especially the migrating birds. The conclusion that feed could be the main source of *MAA* is disproved by the infrequent isolation of *MAA* from the environment i.e. peat and sawdust (Matlova et al., 2004, 2005), which is usually the source of *MAH* infection (Matlova et al., 2005; Trckova et al., 2006a,b).

The most commonly detected RFLP type F found on different farms was also detected in birds and cattle in previous studies (Dvorska et al., 2003, 2004, 2007). Comparison of other pig RFLP types with cattle RFLP types (Dvorska et al., 2004) shows similarity in RFLP types C, D, E, F, N and T. However, these results are not in agreement with Mobius et

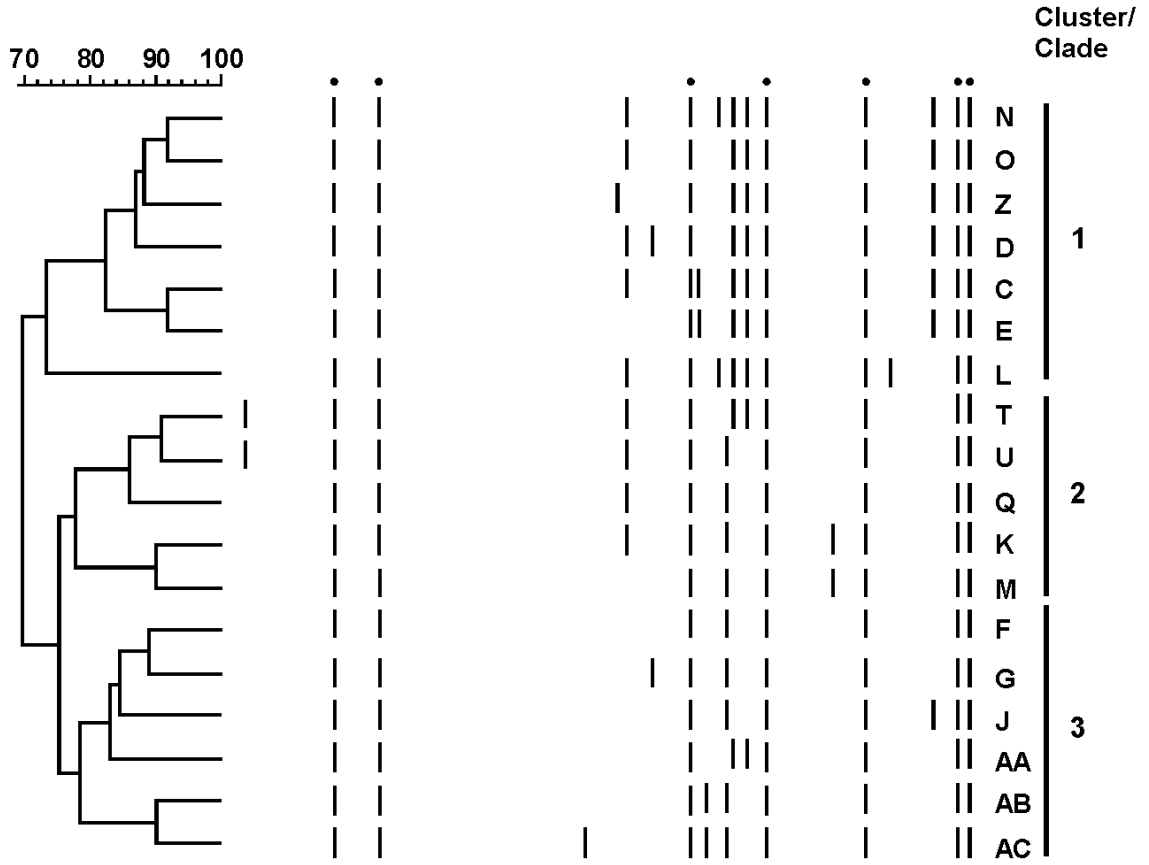

Figure 2. Dendrogram of IS*901* RFLP types after digestion with restriction endonuclease *Pvu*II (the dots show seven identical fragments from various RFLP profiles used as standards to calibrate the results from different gels)

al. (2006), who described host association of *MAA* isolates from cattle and pigs in Germany (Mobius et al., 2006). Comparison of RFLP types found in pigs and birds shows that from both species, strains with RFLP types C, F and G were isolated (Dvorska et al., 2003, 2007). These results indicate an association among pig, cattle and bird isolates and the possibility of an interspecies transmission of infection. Due to the fact that infected birds are often in contact with pigs on the farms, the faeces and bodies of infected birds are likely to be the most common source of avian tuberculosis for pigs.

On the individual farm where the infection was caused by *MAA*, 67.7% of the infections probably originated from one source (water or feed contaminated by infected birds) and was distributed from one pig to another through their faeces (Table 1). Two different RFLP types were present in five (33.3%) of 15 farms with more than one isolate. On three of these farms the two RFLP patterns belonged to the same clade, exhibiting 80% to 90% similarity (Figure 2). Differences in RFLP patterns of isolates from one farm could be explained by two different sources of infection, by mutation of restriction sites surrounding IS*901* and by the transposition and/or the deletion of IS*901*.

However, we did not analyse a representative group of isolates from the Slovenia to compare with isolates from the Czech Republic and the Slovak Republic; we could conclude that our hypothesis about the different pattern of isolates from different countries was not confirmed. Similarly, UPGMA analysis did not reveal any higher similarity among RFLP patterns of isolates from these countries (Figure 2).

Molecular typing methods are very important for understanding of the transmission of the *MAC* infection, but the results obtained must be interpreted with caution and with respect to epidemiological information. The genetic analysis produced by the IS*901* RFLP using only one restriction enzyme seems to have a rather limited discriminatory power; therefore, we recommend to use this method in combination with other molecular methods

e.g. PFGE. In order to exactly identify the sources of *MAA* infection in pigs, detailed and focused studies investigating possibly contaminated environmental specimens will be needed. These studies could lead to new knowledge about the sources and transmission of *MAA* in domestic animals.

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