Genotyping of *Mycobacterium avium* subsp. *avium* isolates from domestic animals in Slovenia by IS*901* RFLP

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ABSTRACT: Apart from birds, *Mycobacterium avium* subsp. *avium* (*MAA*) is often isolated from granulomatous lesions in pigs and occasionally from cattle and other animals. The objectives of this study were the detection of IS*901* restriction fragment length polymorphism (RFLP) types of *MAA* isolates from different species of domestic animals between the years 1998 and 2004 and the comparison of the detected RFLP types with previously described RFLP types collected in the database of the OIE Reference Laboratory for Avian Tuberculosis (Brno, Czech Republic). Furthermore, the RFLP types of the isolates obtained from *MAA* outbreaks on one of the largest pig farms in Slovenia were also investigated. A total of 62 isolates (56 from pigs, five from poultry and one from cattle) were identified with IS*901* PCR and IS*901* RFLP typed using restriction endonucleases *Pvu*II and *Pst*I. Seven *Pvu*II RFLP and 11 *Pst*I RFLP types resulted in 12 combined *Pvu*II *Pst*I types; none of these matched the combined RFLP types described in previous studies. Our contributions to the database were two new *Pvu*II and eight new *Pst*I RFLP types. Identical RFLP types were found among isolates of animals originating from individual farms. Finding of identical RFLP types within a farm is not surprising because the animals were epidemiologically related and infected with one strain. A unique RFLP type F-A17 was detected in isolates from different pig herds and also in isolates from poultry. Detection of identical RFLP types on different farms may reflect one *MAA* source. The other combined *Pvu*II *Pst*I RFLP types were identified only once which indicates considerable variety of *MAA* RFLP types in Slovenia.

Keywords: genotyping; mycobacteriosis; avian tuberculosis; molecular epidemiology; zoonosis; database for IS*901* RFLP types

Mycobacterium avium is currently divided into four subspecies, *M. avium* subsp. *avium* (*MAA*), *M. avium* subsp. *paratuberculosis* (*MAP*), *M. avium* subsp. *silvaticum* (*MAS*) and *M. avium* subsp. *hominissuis* (*MAH*; Thorel et al., 1990; Mijs et al., 2002; Turenne et al., 2007). These organisms range from zoonotic and enzootic pathogens to ubiquitous mycobacteria causing opportunistic infections in a variety of hosts including birds, ruminants, pigs and humans (Pavlik et al., 2000).

MAA is the causative agent of avian tuberculosis; it may infect many animal species but birds (especially poultry) are particularly susceptible to infection which often leads to a fatal outcome (Shitaye et al., 2008a,b, 2009a). In farm animals, particularly in pigs and cattle, *MAA* causes avian tuberculosis with tuberculous lesions mostly localized in the head and mesenteric lymph nodes (Pavlik et al., 2005; Shitaye et al., 2006). Tuberculous lesions have also been found in other tissues, e.g., in the inguinal lymph node of a pig (Trckova et al., 2009) or the respiratory tract of one old horse (Pavlik et al., 2008). *MAH* was proposed to distinguish organisms found in humans and pigs from those isolated from

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birds. Predominantly found in the environment, *MAH* isolates are weakly virulent for birds but are frequently encountered in tuberculous lesions in different animals (especially pigs, occasionally in cattle, deer and other animals) and in humans (Pavlik et al., 2000; Mijs et al., 2002; Shitaye et al., 2006, 2009b; Moravkova et al., 2008).

Complete genome sequences of *MAH* Strain 104 and *MAP* Strain K-10 revealed extensive genomic differences among *M. avium* subspecies. A recently published phylogeny study based on multilocus sequence analysis demonstrated considerable heterogeneity among *MAH* isolates which is not the case for pathogenic subspecies of *M. avium (MAP* and *MAA/MAS)*. This is not surprising considering the opportunities for genetic exchange in diverse environmental habitats in which *MAH* resides (Turenne et al., 2008).

IS*901* RFLP typing is used for the differentiation of *MAA* isolates in spite of its rather limited polymorphism (Ritacco et al., 1998; O'Grady et al., 2000; Dvorska et al., 2003, 2004, 2007; Moravkova et al., 2007; Shitaye et al., 2008a). This is more or less successfully overcome by using a parallel combination of different restriction endonucleases to increase the number of discernable RFLP types. For the past few years, less complex PCR-based mycobacterial interspersed repetitive units-variable-number tandem repeat (MIRU-VNTR) typing has been investigated as an alternative tool for genotyping *M. avium* isolates (Bull et al., 2003; Romano et al., 2005; Thibault et al., 2007; Möbius et al., 2008).

In the past years, mycobacteriosis in pigs has been a common occurrence on both large and small farms in Slovenia. The predominant causative agent in the years 1996 and 1997 was *MAH*, regardless of the farm size (Ocepek and Pate, 2000). In the period 2000–2003, *MAH* was isolated most frequently on small farms in contrast to large farms where *MAA* was diagnosed more often (Pate et al., 2004). A total of 13 *MAA* isolates showed three different *Pvu*II IS*901* RFLP types (Moravkova et al., 2007).

The purpose of this study was the detection of IS*901* RFLP types of *MAA* isolates from different species of domestic animals (mainly pigs) in Slovenia between the years 1998 and 2004, and comparison of the detected IS*901* RFLP types with the types described previously. Furthermore, we wanted to characterize the isolates obtained during outbreaks of avian tuberculosis on one of the largest pig farms in Slovenia.

MATERIAL AND METHODS

Mycobacterial isolates

A total of 62 *MAA* isolates collected between the years 1998 and 2004 from 16 small pig farms, one large pig farm with two herds, five poultry flocks, one cattle farm and two imported pigs were included in the study (Table 1). The majority of isolates originated from pigs (n = 56), five isolates were obtained from poultry and one isolate from cattle. In pigs, the isolates were cultivated from a range of specimens (submandibular, mesenteric and inguinal lymph nodes and liver) in contrast to poultry and cattle specimens, which included only the liver and lungs, respectively. Pig specimens were collected at the slaughterhouses as a result of routine surveillance and tissues with granulomatous changes were sent to the laboratory. Sampling of poultry and cattle tissues was done because the animals showed clinical signs of disease. The geographical origin of the isolates reflects the density of animal husbandry in different parts of Slovenia.

Cattle isolate. The cattle isolate, causing pulmonary disease in a cow, originated from a small farm located in the central part of the country, as described previously (Ocepek et al., 2003).

Pig isolates. Fifteen isolates were collected from the animals from small pig farms (< 1 000 animals) and 39 isolates were obtained from animals originating from one large pig farm (≥ 1 000 animals) with two separate herds A and B located in different regions of Slovenia (Table 1). The stock exchange between these two herds was limited to the transfer of young sows from herd A to herd B for reproductive purposes. Animals in both herds were fed with feeding material of the same origin that was later stored separately in piggeries of different herds. In three cases more than one isolate was cultivated from different tissues of individual pigs. In one pig from a large farm, two isolates were obtained, one from submandibular and the other from mesenteric lymph nodes. From the second pig of the same origin, the two isolates were cultivated from submandibular lymph nodes and a pool of mesenteric and inguinal lymph nodes, respectively. The third case originated from one small farm SF10 where three isolates were obtained from a single pig; one from submandibular lymph nodes and the other two from mesenteric lymph nodes and liver, respectively. The remaining two isolates were obtained from two imported pigs (Table 1).

Table 1. Origin of 62 *Mycobacterium avium* subsp. *avium* isolates investigated with IS*901* RFLP in Slovenia

Farm	Origin of animals			Isolate origin				Number of		Year of isolation
	region			animals			tissue	animals	isolates	
	central	east	south-east	cattle	pigs	poultry				
SF1	+			+			lungs	1	1	2000
SF2		+			+		LN	1	1	2000
SF3		+			+		LN	1	1	2000
SF4			+			+	liver	1	1	2000
SF5			+			+	liver	1	1	2000
SF6		+			+		LN	1	1	2001
SF7			+			+	liver	1	1	2001
SF8		+			+		LN	1	1	2001
SF9			+			+	liver	1	1	2002
SF10		+			+		LN, liver	4	6	2004
SF11			+			+	liver	1	1	2001
SF12		+			+		LN	1	1	2001
SF13		+			+		LN	1	1	1999
SF14		+			+		LN	1	1	1999
SF15		+			+		LN	1	1	1999
SF16			+		+		LN	1	1	1999
LF1-A	+				+*		LN, liver	21	23	1998, 2001–2003
LF1-B			+		+*		LN	16	16	2001, 2003
I1		+			+		LN	1	1	2003
I2		+			+		LN	1	1	1998
Total	2	11	7	1	13*	5	3 tissues	58	62	7-year period

SF = small farm (< 1 000 animals); LF = large farm (≥ 1 000 animals); I = imported animal; LN = lymph nodes; A = herd A; B = herd B; *large pig farm LF1 with two herds A and B is considered as only one farm

Poultry isolates. Poultry isolates originated from five extensively bred flocks. No data on possible epidemiological connections among the flocks were available.

Identification of the isolates

Isolates were cultured on Löwenstein-Jensen, Stonebrink and Middlebrook 7H10 media and identified with IS*901* PCR using primers IS*901*-1 (5'-GCA ACG GTT GTT GCT TGA AA-3') and IS*901*-2 (5'-TGA TAC GGC CGG AAT CGC GT-3') described previously (Kunze et al., 1992). Amplification products of 1108 bp were analysed by electrophoresis on 2% agarose gels and detected by ethidium bromide staining.

RFLP analysis

The method was performed according to previously published parameters (van Soolingen et al., 2002; Dvorska et al., 2003) with slight modifications. Bacterial cultures were resuspended in Tris-EDTA (TE) buffer. Cells were lysed with lysozyme, sodium dodecyl sulphate (SDS) and proteinase K. Cell wall debris, denatured proteins and polysaccharides were complexed to cetyltrimethylammonium bromide (CTAB) and removed by centrifugation.

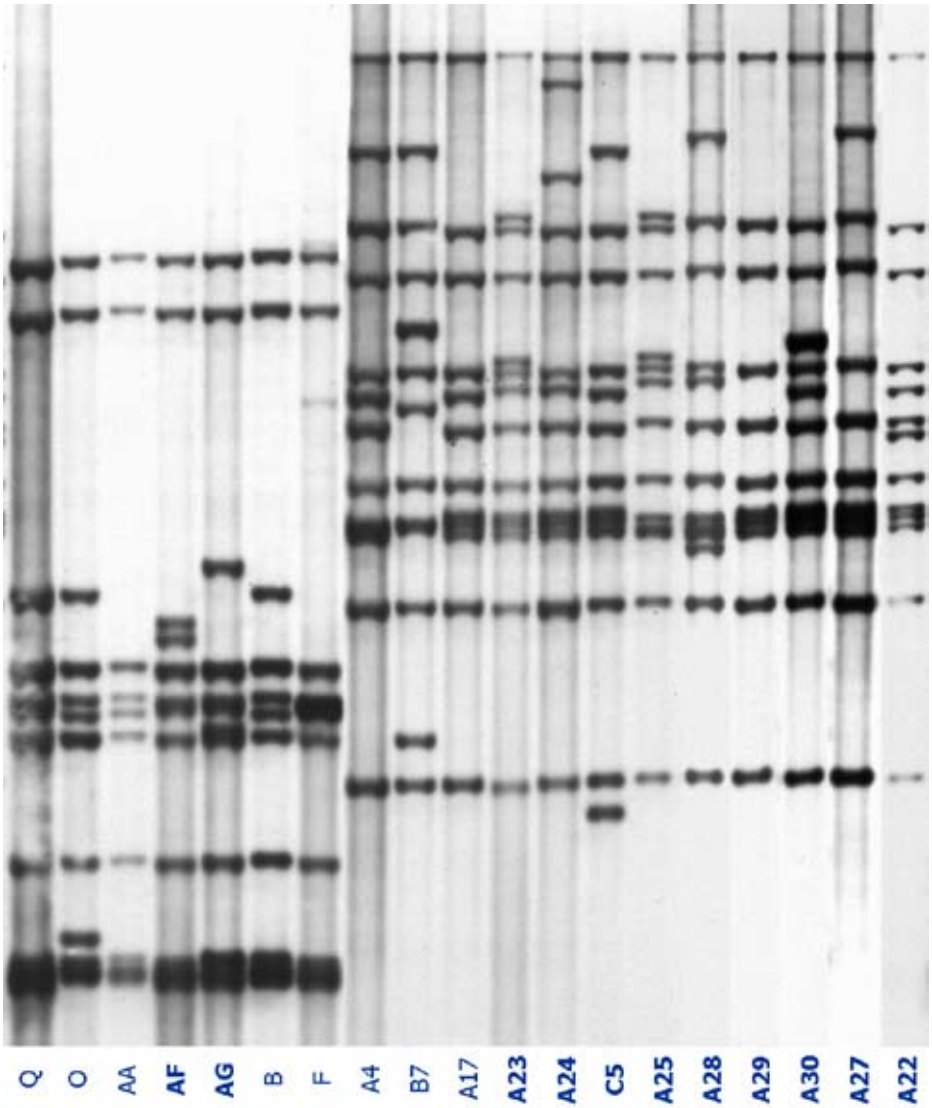

Figure 1. IS*901* RFLP types discovered in 62 *Mycobacterium avium* subsp. *avium* isolates in this study: *Pvu*II RFLP types Q to F and *Pst*I RFLP types A4 to A22. New RFLP types are in bold. RFLP types are designated according to Dvorska et al. (2003; VRI designation system). Reference *Mycobacterium avium* subsp. *avium* strain R13 showed the *Pvu*II *Pst*I RFLP type F-A22

DNA was extracted with chloroform/isoamylalcohol, precipitated with isopropanol and dissolved in TE buffer. The DNA concentration was evaluated semi-quantitatively by visual comparison with standards after electrophoresis on a 0.8% agarose gel. DNA was then digested in parallel by the restriction endonucleases *Pvu*II (Sigma Aldrich, Saint Louis, MO, USA) and *Pst*I (Promega, Madison, Wi, USA). DNA fragments were separated by electrophoresis (50 V, overnight) in 0.8% agarose gels and then vacuum blotted (Biometra, Göttingen, Germany) onto nylon membranes (Hybond-N+, Amersham Biosciences, Buckinghamshire, UK).

The hybridisation probe consisted of a 1 108 bp PCR product, amplified using previously described primers (Kunze et al., 1992), purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and labelled using the ECL direct nucleic acid labelling and detection system (Amersham Biosciences, Buckinghamshire, UK). The membranes were hybridised at 42°C overnight. RFLP types obtained on an ECL Hyperfilm (Amersham Biosciences, Buckinghamshire, UK) were scanned (Model GS-700 Imaging Densitometer, BioRad, Hercules, CA, USA) and analysed by the BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Reference *MAA* Strain R13 was used for band normalisation. Dendrograms with 1.2% tolerance were created using a UPGM (Dice coefficient) algorithm generated by BioNumerics software.

Designation of RFLP types

The RFLP types described herein were designated according to the extensive IS*901* RFLP-based study of *MAA* isolates performed by Dvorska et al. (2003) who described 25 *Pvu*II (designated alphabetically from A to Y), 25 *Pst*I (designated as A1-16, B1-2, C1-3, D1 and L1-3) and 52 combined *Pvu*II *Pst*I RFLP types. Since then, several additional RFLP types have been discovered and collected in a database at the Veterinary Research Institute (VRI), Brno (Czech Republic), but have not yet been published. This database includes IS*901* RFLP types of *MAA* isolates originating from some central European countries. Therefore, our results, based on the visual comparison of our RFLP types with the previously described RFLP types, were sent to the VRI in order to check them, to update the collection and to name new RFLP types. The nomenclature of the RFLP types is thus in concordance with the nomenclature established and used at the VRI.

RESULTS

Detected IS*901* RFLP types

All 62 isolates were successfully digested with restriction endonuclease *Pvu*II and seven distinct RFLP types with up to 11 bands were observed showing an average similarity of 83% (Figures 1 and 2A).

Typing with restriction endonuclease *Pst*I was successfully accomplished in 52 (83.9%) isolates, resulting in 11 distinct *Pst*I RFLP types, which in general consisted of more bands (up to 14) than *Pvu*II RFLP types and expressed an average similarity of 75.9% (Figures 1 and 2B). Digestion with restriction endonuclease *Pst*I was found to be rather problematic, especially for the cultures grown on Middlebrook 7H10 medium. In order to obtain interpretable digestion results it was sometimes necessary to subculture the isolate and to repeat the DNA extraction and digestion (data not shown).

A combination of the *Pvu*II *Pst*I RFLP types obtained by the parallel digestion of the samples with restriction endonucleases *Pvu*II and *Pst*I resulted in 12 *Pvu*II *Pst*I RFLP types Q-A4, Q-A27, O-B7, F-A17, F-A29, B-C5, B-A28, AG-A24, AF-A23, AF-A25, AA-A17 and AA-A30. In 10 isolates with *Pvu*II RFLP types AA, F and O, the digestion with restriction endonuclease *Pst*I failed (Table 2).

Inside one large pig farm with two individual herds (LF1-A and LF1-B) and in one small pig farm (SF10) isolates with identical RFLP types O-B7, AA-A17 and B-C5 were found, respectively. RFLP type F-A17 was detected in single isolates from different small pig farms and in one poultry flock; the other RFLP types were unique (Table 2).

Isolates collected from different tissues of a single animal shared identical RFLP types. Four isolates of two pigs from large farm LF1-A showed RFLP type O-B7 while three isolates of a pig from small farm SF10 shared RFLP type B-C5 (Table 2).

Regarding geographical origin, isolates with identical and different RFLP types were found within individual regions. In the central region, including one cattle farm and herd A from a large pig farm, different RFLP types were detected. The same applies for the south-eastern region where five poultry flocks and one small pig farm were located. In the eastern region, in which the highest number of (exclusively pig) farms were investigated, both identical and different RFLP types were observed (Tables 1 and 2).

Comparison of detected RFLP types with the VRI database patterns

Five *Pvu*II RFLP types AA, B, F, O and Q matched previously discovered RFLP types while two *Pvu*II RFLP types AF and AG have never been submitted to the VRI database before. Three *Pst*I RFLP types A4, A17 and B7 matched previously discovered RFLP types collected in the VRI database while eight *Pst*I RFLP types A23, A24, A25, A27, A28, A29, A30 and C5 were found to be new. None of *Pvu*II *Pst*I RFLP types described herein matched the combined RFLP types found in the studies of Dvorska et al. (2003, 2004, 2007). Among *MAA* isolates from Slovenia genotyped by Moravkova et al. (2007), one isolate was of *Pvu*II RFLP type M that was not found in the present study, while the remaining investigated isolates expressed *Pvu*II RFLP types O and AA that were detected also in this study.

Epidemiological results

Isolates with *Pvu*II RFLP types O and AA were found on the farm with the outbreaks of avian tuberculosis. RFLP type O was found in 23 isolates from pigs originating from herd A of a large farm located in the central part of the country, while

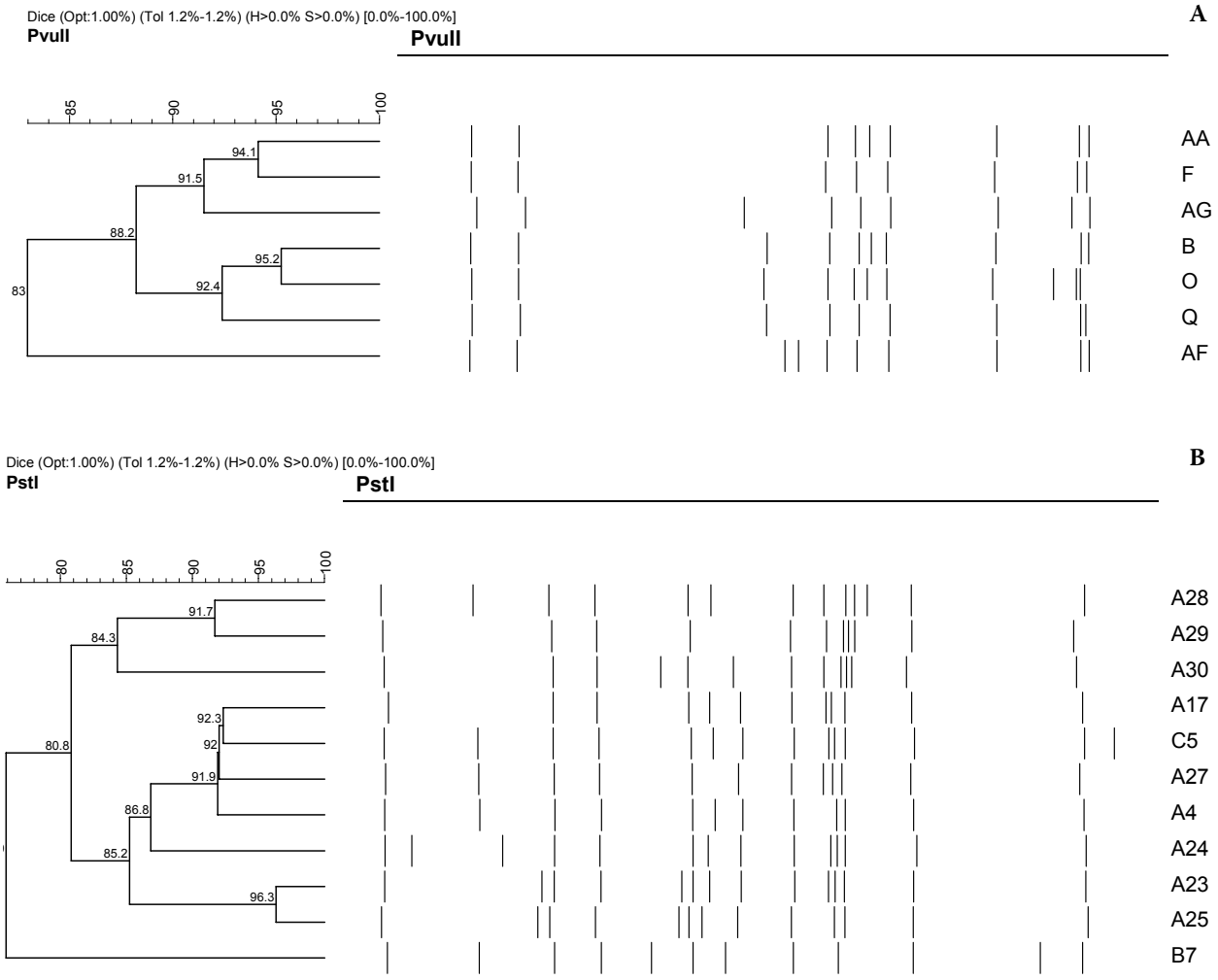

Figure 2. A = dendrogram of *Pvu*II IS*901* RFLP types of *M. avium* subsp. *avium* isolates; B = dendrogram of *Pst*I IS*901* RFLP types of *M. avium* subsp. *avium* isolates

RFLP type AA was found in 17 isolates from pigs originating from herd B of the same farm, located in the south-eastern part of the country. All isolates with *Pvu*II RFLP type O exhibited the same *Pst*I RFLP type B7. The same applies for RFLP type AA, which had *Pst*I RFLP type A17 with the exception of one *Pst*I RFLP type A30. *Pst*I RFLP types could not be determined for four isolates (one *Pvu*II RFLP type AA and three *Pvu*II RFLP types O). The same RFLP types were detected in individual herds at different time periods (Table 2).

DISCUSSION

Mycobacteriosis in pigs is a common occurrence. Even though the first IS-based studies of *M. avium* isolates reported no IS*901*+ strains in pigs (Bono et al., 1995; Nishimori et al., 1995), anyhow these animals are susceptible to infection with both *MAA* and *MAH*. The prevalence seems to be correlated with the presence of certain subspecies in the environment. Later studies (Ahrens et al., 1995; Thegerström et al., 2005) reported almost equal proportions of *MAA* isolates from investigated pigs (50% and 46%, respectively). In Slovenia, *MAA* in pigs was determined to have a 38% prevalence during the years 1996–1997 (Ocepek and Pate, 2000) and one of 33.8% in the period 2000–2003 (Pate et al., 2004).

Selection of the same restriction endonucleases that have been used in the most extensive study on *MAA* genotyping by IS*901* RFLP published so far (Dvorska et al., 2003) enabled us to achieve comparable results. Despite being rather problematic, digestion with *Pst*I revealed a number of new RFLP types and this contributed to a greater diversity of RFLP types. *Pst*I RFLP types of our isolates were

Table 2. *Pvu*II *Pst*I IS*901* RFLP types of *Mycobacterium avium* subsp. *avium* isolates from domestic animals in Slovenia

RFLP type[a]		Origin and number of isolates				Farm[b]
*Pvu*II	*Pst*I	cattle	pigs	poultry	total	
AA	nd	0	1	1	2	LF1-B, SF7
AA	A17	0	14	0	14	LF1-B
AA	A30	0	1	0	1	LF1-B
AF	A23	0	1	0	1	SF3
AF	A25	0	1	0	1	SF8
AG	A24	0	1	0	1	SF2
B	A28	0	1	0	1	I2
B	C5	0	6	0	6[c]	SF10
F	nd	0	3	2	5	SF5, SF6, SF9, SF15, SF16
F	A17	0	3	1	4	SF4, SF12, SF13, SF14
F	A29	0	0	1	1	SF11
O	nd	0	3	0	3	LF1-A
O	B7	0	20	0	20[d]	LF1-A
Q	A4	0	1	0	1	I1
Q	A27	1	0	0	1	SF1
Total						
7	11	1	56	5	62	19

nd = not detected due to failed digestion with restriction endonuclease *Pst*I

[a]nomenclature of the profiles is in concordance with the nomenclature established and used at Veterinary Research Institute Brno, Czech Republic (Dvorska et al., 2003)

[b]farm origin: see in Table 1

[c]the sum includes three isolates from different tissues of a single animal

[d]the sum includes four isolates from two different tissues of two individual animals (from each animal two isolates from two different tissues were obtained)

mostly regarded as new as they were not found in the VRI database (Figure 1; Dvorska et al., 2003).

Pig isolates from the small farms exhibited a certain range of genetic diversity as a different *Pvu*II *Pst*I RFLP type was detected on almost each farm. The majority of investigated isolates originated from the eastern part of Slovenia, which reflects the highest number of piggeries in this region. Different RFLP types discovered in the same region suggest the existence of several *MAA* reservoirs. Only isolates with *Pvu*II RFLP type F were detected on more small farms. The same situation was observed in the studies of Dvorska et al. (2003, 2004, 2007) and Moravkova et al. (2007) when RFLP type F was detected in birds, cattle and pigs from different areas and countries.

Isolates from poultry flocks mostly had the same *Pvu*II RFLP type F with the exception of one isolate from flock SF7 which shared the RFLP type AA with a number of pig isolates. *Pst*I digestion succeeded in only two cases, resulting in two combined RFLP types: RFLP type F-A29 was unique in our collection while RFLP type F-A17 was discovered also in pig isolates (Table 2). The occurrence of identical RFLP types in different animal species may indicate a transmission of the bacteria. However, an epidemiological link would be needed to prove it in our case. Previously it has been suggested that, especially in the case of small farms with extensive breeding systems, the co-existence of different domestic animals plays a role in the transmission of *MAA* from wild birds to poultry and other species, e.g., pigs (Ocepek, 1996). Regarding the geographical origin of the poultry isolates, it may be concluded that in the south-eastern region of Slovenia avian tuberculosis

was diagnosed in poultry flocks, especially during the period 2000–2002 (Table 1). We have not found the explanation for this situation.

*Pvu*II RFLP type Q of the cattle isolate matched the RFLP type of the isolate I1 from one pig imported from a neighbouring country, but they did not share the same *Pst*I RFLP type: the isolate from the cow was of RFLP type Q-A27 and the isolate from the imported pig was of RFLP type Q-A4 (Tables 1 and 2). This case clearly demonstrates the advantages of parallel digestion by both restriction endonucleases in terms of increasing the discriminatory power (Dvorska et al., 2003).

Investigation of different tissues from a single animal revealed isolates with identical RFLP types. This is not surprising, considering that *MAA* is a professional pathogen. In contrast, investigation of *MAH* infections more often results in cultivation of different isolates from one animal (polyclonal infection) as a consequence of a wide spectre of *MAH* isolates present in the environment (Pate et al., 2008).

The retrospective investigation of *MAA* outbreaks on one of the largest pig farms (with geographically separated herds A and B) in Slovenia revealed that the animals affected in individual herds were infected with isolates of different RFLP types (RFLP type O-B7 in herd A and RFLP types AA-A17 and AA-A30 in herd B), which suggests different sources of infection. However, there was one exception in herd B, where one isolate differed in the *Pst*I RFLP type A30 from the other 14 isolates of *Pst*I RFLP type A17 (Table 2 and Figure 2B). This one band difference could be caused by a transposition or change in restriction sites.

Both pig herds A and B could have been infected by free-living birds or through feed contaminated by bird droppings during storage. However, the infection could have been acquired also through contaminated bedding material. As possible environmental sources were not investigated for the presence of mycobacteria because of the retrospective nature of the study, we can only speculate about the source of infection. The occurrence of isolates with identical RFLP types in the years prior to the outbreaks may be explained by the persistence of these RFLP types in the farm environment.

Considering the small number of investigated epidemiologically unrelated isolates, this study revealed a considerable variety of RFLP types as the majority of detected RFLP types were unique. Analysis of more isolates of different origin would most probably add to the heterogeneity of IS*901* RFLP types. This is supported also by the results of previous genotyping studies of animal *MAA* isolates in Slovenia (Ocepek et al., 1998; Moravkova et al., 2007) which demonstrated some *Pvu*II IS*901* RFLP types not detected in the current study.

Finally, we would like to encourage the establishment of an international computerized database of *MAA* RFLP types. Computer-assisted analysis would undoubtedly facilitate comparison and reduce the risk of misidentification of new RFLP types. In addition, a database containing RFLP types of isolates from different sources and geographical regions would facilitate the development of a global prospective on the epidemiology of *MAA* infections.

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