A mixed infection of *Mycobacterium avium* subsp. *paratuberculosis* and *M. a. hominissuis* in one red deer (*Cervus elaphus*) studied by IS*900 Bst*EII and IS*1245 Pvu*II RFLP analyses: a case report

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ABSTRACT: A mixed infection with *Mycobacterium avium* subsp. *paratuberculosis (MAP)* and *Mycobacterium avium* subsp. *hominissuis (MAH)* in one naturally infected red deer stag from a game park is described. The animal was euthanized because of symptoms of poor condition, weight loss and chronic diarrhoea. In spite of that, pathological lesions were observed only in the mesenteric lymph nodes, which were five to ten times enlarged with confluent caseous granulomas of 1 to 10 mm in size. Mycobacteria were isolated from all studied samples: a mixed infection of *MAP* and *MAH* was confirmed by multiplex PCR for the detection of IS*900*, IS*901*, IS*1245* and *dnaJ*. *MAP* of the identical IS*900 Bst*EII RFLP type C1 was isolated from all tissue samples and faeces. *MAH* isolates were detected in six examined tissue samples, including three mesenteric lymph nodes with caseous granulomas. Only minor differences in the band numbers and position of four different IS*1245 Pvu*II RFLP patterns of *MAH* isolates were found. It follows from these results that red deer may potentially be infected with *MAH*, when a *MAP* infection is under way.

Keywords: Johne's disease; mycobacteriosis; game park; molecular epidemiology; zoonosis; food safety

The most significant mycobacterial diseases of free living and farmed red deer (*Cervus elaphus*) are bovine tuberculosis caused by *Mycobacterium bovis*, avian tuberculosis caused by *M. avium* subsp. *avium (MAA)* and paratuberculosis (Johne's disease) caused by *M. a. paratuberculosis* (*MAP*; Mackintosh et al., 2004).

Due to the rapid development of deer farming and the domestication of wild ruminants world-wide during the last three decades, in countries where bovine tuberculosis has been eradicated paratuberculosis has become one of the most important diseases with a big economical impact for deer farmers (Machackova et al., 2004).

Paratuberculosis is an infectious chronic intestinal disease of ruminants. The infection could be introduced to the deer farm by contact with infected faeces from sheep, cattle, or other wildlife ruminants. Paratuberculosis has also been diagnosed in a number of wild species, other than ruminants such as foxes and hares. These animals can serve as a reservoir for *MAP* and potential vectors of the disease (Pavlik et al., 2000; Beard et al., 2001). In contrast to clinical symptoms of the disease in cattle, paratuberculosis in deer may often be clinically diagnosed in young animals, in which severe emaciation and occasionally mortality are observed. Older animals appear to become more resistant to the infection and

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are less susceptible to the disease (Mackintosh et al., 2004; Machackova-Kopecna et al., 2005).

For a better understanding of the transmission and knowledge about the source of *M. avium* species infections in deer, several molecular typing methods have been standardised and applied (Pavlik et al., 2000; Machackova-Kopecna et al., 2005; Glawischnig et al., 2006).

The aim of this study was to describe a case of a red deer infected with both *MAP* and *MAH* and to apply the molecular methods for the typing of *MAP* and *MAH* isolates by insertion sequences (IS) IS*900* and IS*1245* RFLP analyses.

MATERIALS AND METHODS

Anamnestic data. In this study, one red deer stag from a game park in the Czech Republic, at the age of 16 months, was culled due to its poor condition: weight loss and signs of chronic diarrhoea were the observed symptoms.

Pathological examination. Lungs, spleen, liver and the gastrointestinal tract were necropsied.

Microscopic examination. Imprint preparations were made of tissue samples. After fixation by a flame, they were stained by the Ziehl-Neelsen (Z-N) method for the detection of acid-fast rods (AFRs). At least 100 fields of view were examined in each sample under the immersion (magnification 1 000 ×, Olympus BX41).

Culture examination. The tissue and faecal samples were decontaminated with 0.75% HPC (hexa-decyl-pyridinium chloride: cetylpyridinium chloride, Merck No 102340) and cultured on three Herrold Egg Yolk Media (HEYM) with Mycobactin J and incubated at 37°C for up to 12 months according to the previously published procedure (Machackova et al., 2004). Pure cultures were obtained after the subculture and purification of cultures with a mixed infection.

Identification of mycobacterial isolates by PCR. Isolates from the primocultures were identified by a modified PCR targeting the gene for 16S rRNA and by multiplex PCR (Moravkova et al., 2008). A modified PCR for 16S rDNA enables the detection of the genus *Mycobacterium* and distinguishes between *M. avium* species and other mycobacteria. Multiplex PCR enables the detection of *MAP* (*dna*J+, IS*900*+, IS*901*– and IS*1245*–), *MAA* (*dna*J+, IS*900*–, IS*901*+ and IS*1245*+) and *MAH* (*dna*J+, IS*900*–, IS*901*– and IS*1245*+) in one PCR reaction.

RFLP analysis and probe preparation. The IS*1245* RFLP analysis of *MAH* isolates was conducted according to the method described previously (van Soolingen et al., 1998). *MAP* isolates were differentiated by IS*900* RFLP analysis described previously (Pavlik et al., 1999). Isolated DNA from *MAH* was digested with restriction endonuclease *Pvu*II and DNA isolated from *MAP* was digested with restriction endonuclease *BstE*II.

Probes were prepared by the PCR amplification of DNA from *MAP* or *MAH*. For the amplification of IS*1245,* the following primers were used: 5′-GCC GCC GAA ACG ATC TAC-3′ and 5′-AGG TGG CGT CGA GGA AGA-3′ (Guerrero et al., 1995). For the amplification of IS*900,* the following primers were used: 5′-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3′ and 5′-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3′ (Whittington et al., 1998). Labelling was performed using the ECL Direct Labelling Kit (Amersham, UK). IS*900* RFLP profiles were evaluated according to a system described by Pavlik et al. (1999).

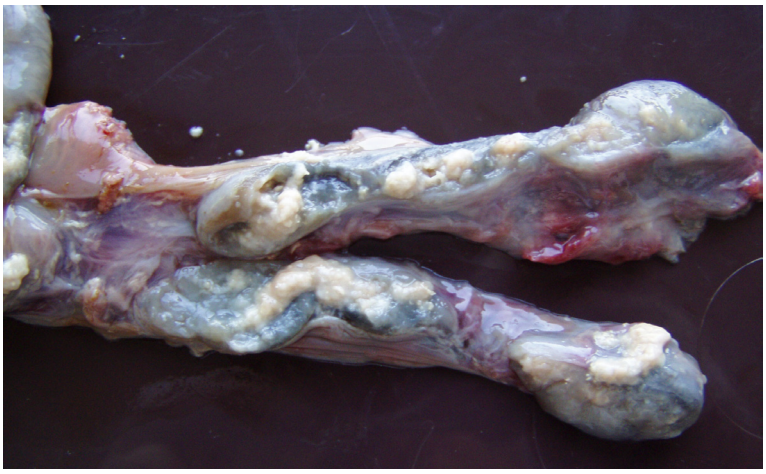

Figure 1. Enlarged mesenteric lymph nodes full of lymphatic fluid, with confluent granulomas originated from one naturally infected red deer

RESULTS

Pathological examination revealed five to ten times enlarged mesenteric lymph nodes full of lymphatic fluid, with confluent caseous granulomas between 1 and 10 mm in size in the cortex (Figure 1). The rest of the lymph nodes were of normal size and state. AFRs were observed after Z-N staining using the light microscope (Olympus BT17, Japan) in a massive quantity in all examined tissue samples (liver, spleen and intestinal tract) except for the lungs and lung lymph nodes. By culture, mycobacteria were observed after two months of growth on the HEYM Mycobactin J from all examined samples (Table 1).

All twelve isolates were identified as *M. avium* species by PCR for 16S rRNA. In six isolates, multiplex PCR revealed both *MAP* and *MAH* and in the other six isolates only *MAP* (Table 1).

All twelve *MAP* and six *MAH* isolates from different sections of the gastrointestinal tract and from faeces were analyzed by the IS*900* and IS*1245* RFLP method, respectively. All *MAP* isolates were of the identical IS*900* RFLP type C1 according to Pavlik et al. (1999). *MAH* isolates were of four different IS*1245* RFLP types designated as A (n = 2), B (n = 1), C (n = 2) and D (n = 1). The transpositions of one to three fragments were observed (Figure 2).

DISCUSSION

Infections caused by *MAH* and *MAA* are largely responsible for the high level of sensitization to avian purified protein derivatives, used for the tuberculin skin test for deer, but infections are usually subclinical (de Lisle et al., 1995). In a few cases, lesions in retropharyngeal, mesenteric and ileocaecal lymph nodes have been found. Occasionally, clinical signs developed in animals under stressful conditions and when their immune system was compromised (Mackintosh et al., 2004). However, it is impossible to discriminate between infections of *MAP*, *MAA* and *MAH* only according to pathological lesions.

In this study, we have described a mixed infection with two mycobacterial subspecies, *MAP* and *MAH,* in one naturally infected red deer from a game park. The animal suffered typical clinical signs of *MAP* infection, which are diarrhoea and

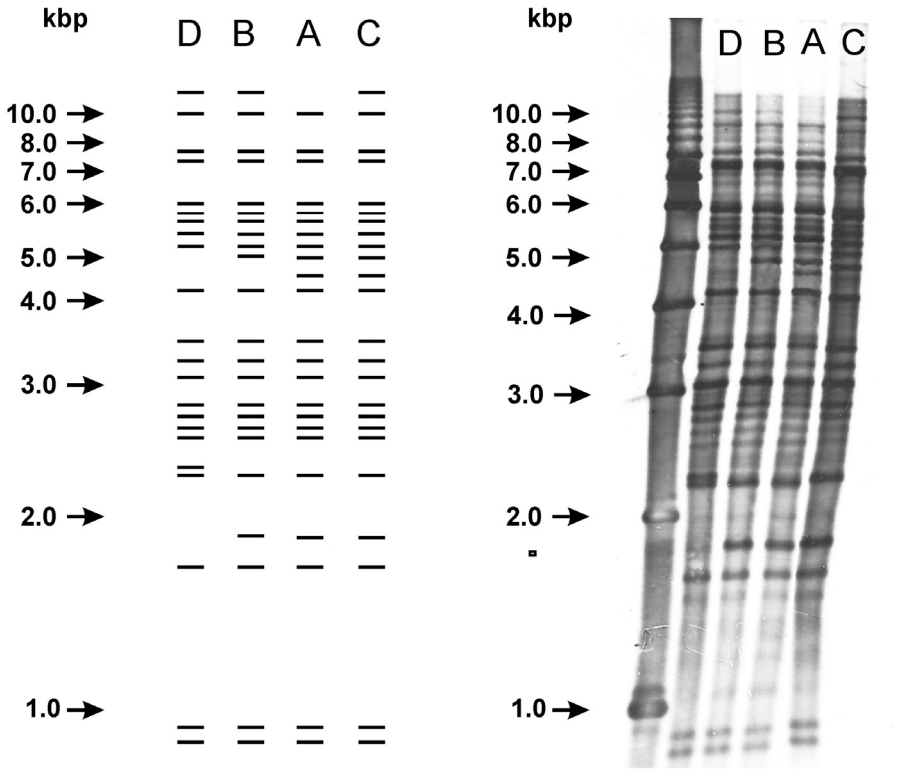

Figure 2. Four IS*1245* RFLP patterns of *Mycobacterium avium* subsp. *hominissuis* isolates after digestion with restriction endonuclease *Pvu*II. A 1 kbp ladder (ABGene, United Kingdom) is situated on the left side of the patterns

Table 1. Examined tissue samples from one naturally infected red deer (*Cervus elaphus*)

Examined samples		Laboratory examination			PCR from primocultures[d]				RFLP profile[e]	
System	Tissue	P-A[a]	AFRs[b]	culture[c]	*dnaJ*	IS*900*	IS*1245*	IS*901*	IS*900*	IS*1245*
Gastrointestinal tract	jejunal mucosa (beginning)	–	+++	+++	+	+	+	–	C1	A
	jejunal lymph node (beginning)	+	+++	++	+	+	+	–	C1	B
	jejunal mucosa (end)	–	+++	+++	+	+	–	–	C1	
	jejunal lymph node (end)	+	+++	++	+	+	+	–	C1	A
	ileocaecal mucosa	–	+++	+	+	+	+	–	C1	C
	ileocaecal lymph node	+	+++	++	+	+	+	–	C1	C
	faeces	nt	nt	+++	+	+	–	–	C1	
Parenchymatous organs	liver tissue	–	+++	+++	+	+	–	–	C1	
	liver lymph node	–	+++	+++	+	+	+	–	C1	D
	spleen	–	+++	+++	+	+	–	–	C1	
	lung tissue	–	–	+	+	+	–	–	C1	
	lung lymph node	–	–	+	+	+	–	–	C1	

[a]pathological finding: – = without lesions, + = five to ten times enlarged and full of lymphatic fluid with solely and confluent granulomas

[b]acid-fast rods (AFRs) detected after the Ziehl-Neelsen staining: +++ = (massive positivity with thousands AFRs at least 200 microscopic fields of view for each sample)

[c]isolation on Herrold Egg Yolk Medium with Mycobactin J: + = 1 to 20 colony forming units (CFU), ++ = 21 to 100 CFU, +++ = ≥100 CFU

[d]targeted genes detected by multiplex PCR described previously (Moravkova et al., 2008)

[e]IS*900* RFLP analyses carried out according to standardized method (Pavlik et al., 1999) and IS*1245* RFLP analysis done according to the previously published protocol (van Soolingen et al., 1998)

nt = not tested

weight loss. Pathological examinations revealed greatly enlarged jejunal lymph nodes with confluent granulomas (Figure 1). Similar pathological lesions were also described in other deer infected with *MAA* or with *MAP* and *MAH* (Glawischnig et al., 2006).

Diagnosis of *MAP* is usually based on Mycobactin J dependence on HEYM, but it was described that this should be interpreted with caution. Some *MAP* isolates are not strictly dependent on Mycobactin J (Motiwala et al., 2004) and, in some cases, a mixed infection of *MAP* and members of the *M. avium* complex may appear (Glawischnig et al., 2006). Due to the fact that sometimes it is not possible to extract DNA from one single colony forming unit, *MAA* and *MAH*, in a mixed infection including *MAP*, may be overlooked when using PCR specifically based on the identification of *MAP* only (Godfroid et al., 2005).

In our study we have used the multiplex PCR for *dna*J, IS*900*, IS*901* and IS*1245* that enables the detection of *MAP*, *MAA*, or *MAH* in one PCR reaction. This enables the differentiation of a single infection from a mixture of *MAP* and *MAA* or *MAP* and *MAH* (Moravkova et al., 2008). As mentioned by some authors, IS*900*-like elements are present in mycobacterial species other than *MAP* (Englund et al., 2002) and they advise using another target sequence, for instance f*57* or IS*Mav*2 (Poupart et al., 1993; Strommenger et al., 2001). To avoid the generation of false-positive results, we have performed another 16S rRNA-PCR described previously by Wilton and Cousins (1992) and modified by Moravkova et al. (2008). This PCR is not based on a specific fragment for *MAP,* but enables the identification of *Mycobacterium* sp. (1 030 bp size amplification product) and simultaneously distinguishes *M. avium* (180 bp size amplification product) from other opportunistic mycobacteria.

Methods for the differentiation or subtyping of bacterial strains provide important information for molecular epidemiological analysis and help the understanding of bacterial genetics. In our study, IS*900* RFLP analysis of *MAP* isolates, from different locations of one animal revealed that all isolates were of RFLP type C1. These results are in agreement with other studies where IS*900* RFLP patterns are described as very stable over a long period of time (Cousins et al., 2000; Ayele et al., 2004). Since RFLP type C1 is the predominating pattern of *MAP* isolates from deer and cattle from the Czech Republic (Pavlik et al., 2000; Machackova et al., 2004; Machackova-Kopecna et al., 2005), it is not possible to conclude, without epidemiological knowledge, if *MAP* was transmitted from only one source of infection.

Using IS*1245* RFLP analysis, variations of one to three copies were observed between patterns for *MAH* isolates in this red deer (Figure 2). These minor variations in the isolates of the single strain from one animal or patient were also described by other authors (Pestel-Caron and Arbeit, 1998). Differences in the band pattern were also observed following cultivation in liquid media 33 times over a period of one year (Bauer and Andersen, 1999). These relatively minor differences in the fingerprint pattern are thought to be caused by either a mutation eliminating the restriction site or loss of an IS*1245* copy.

As mentioned above, *MAP* is one of the most common causal agents of mycobacterial infection in deer and also in other ruminants (Ayele et al., 2001). *MAP* has been isolated from bulls not only from the gastrointestinal tract, but also from other different organs including liver, genital organs and different lymph nodes (Ayele et al., 2004), and from red deer from hepatic lymph nodes and spleen (Machackova-Kopecna et al., 2005). On the other hand, *MAH* is prevalent in the environment and causes economic loses in domestic pigs (Pavlik et al., 2005; Shitaye et al., 2006). However, in deer, only a few cases of *MAH* have been documented (Wards et al., 1991; Glawischnig et al., 2006) and these cases were usually connected with some other infection, e.g., *MAP*.

Due to this fact, we suppose that the red deer was first infected with *MAP* and that following this infection, the "immunocompromised" animal was consecutively infected with *MAH* from one single source. This *MAH* infection primarily infected the gastrointestinal tract, probably caused the granulomatous inflammation (Figure 1), and *MAH* was then spread through the blood circulation and was captured in liver lymph nodes (Table 1). This hypothesis is supported by the only minor difference in the number of bands in the RFLP pattern of *MAH* isolates from different organs (Figure 2).

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