Distribution and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in farmed red deer (*Cervus elaphus*) studied by faecal culture, serology and IS*900* RFLP examinations

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ABSTRACT: The objectives of this study were the determination of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) distribution in organs of farmed red deer (*Cervus elaphus*) and the investigation of its vertical and horizontal spread among animals, using serology, cultivation and the standardized IS*900* RFLP method. During the three year of study, the production of antibodies for *MAP* increased from 0 in the first year to 7.7% (positive) and 0 to 88.5% (dubious) in the third year of the study. The first performed global culture examination of faecal samples from 28 animals was negative for *MAP*. In the three subsequent examinations of animals, the following positivity was found: 5.9%, 34.6%, and 36.8%, respectively. In the last year of the study, clinical signs such as diarrhoea were observed in four animals. The animals with clinical symptoms and those that were found to be infected with *MAP* by serology or faecal culture were euthanized. *MAP* was isolated from the intestinal tract and pulmonary lymph (tracheobronchial or mediastinal lymph nodes) nodes of all studied animals. Apart of this *MAP* was also isolated from reproductive organs, such as the mammary gland, milk, uterus, amniotic fluid and testicles. Application of the IS*900* RFLP method revealed that the prevailing *MAP* isolates were of RFLP type B-C1; this profile was found in all types of tissue samples as well as in faeces, milk and amniotic fluid. In five animals a mixed infection of two profiles B-C1 and B-C5 or B-C1 and B-C16 was detected.

Keywords: Johne's disease; epidemiology; Crohn's disease; food safety; ecology

Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), is generally regarded as a disease of domestic ruminants, but it also affects other ruminant species such as red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). *MAP* is not only shed by infected animals, but may also be present in intestinal parasites of sheep (Whittington et al., 2001) or be shed by different invertebrates (Fischer et al., 2003, 2004). *MAP* cannot propagate outside a host organism (Ayele et al., 2001) but is fairly resistant to the challenges posed by different constituents of the external environment (Richards, 1981; Whittington et al., 2004).

Due to the development and intensification of the deer farming in Europe (Power et al., 1993; Fawcett et al., 1995; Machackova et al., 2004) and worldwide (DeLisle et al., 1993; Manning et al., 1998; Mackintosh et al., 2004; O'Brien et al., 2006), the prevalence of paratuberculosis in these ruminants has recently increased (Machackova et al., 2004; Mackintosh et al., 2004). However, paratuberculosis in free living ruminants has not been as extensively studied as in cattle (Ayele et al., 2001).

Partially supported by the Brussels Grant No. FOOD-CT-2005-007081 (PathogenCombat) and by the Ministry of Agriculture of the Czech Republic (Grants No. MZE 0002716201 and No. QH81065).

The most common way of *MAP* transmission in cattle is by consumption of infected milk/colostrum, faecal contamination of teats or ingestion of contaminated feed and water (Taylor et al., 1981; Sweeney et al., 1992a; Streeter et al., 1995). Vertical transmission of *MAP* to the foetus *in utero* (Seitz et al., 1989; Sweeney et al., 1992b) and the potential transmission of *MAP* in bull semen (Larsen et al., 1981; Ayele et al., 2004) has also been reported. Distribution of *MAP* in the organisms has been studied in different age categories of cattle of various breeds naturally infected with paratuberculosis. *MAP* in cows has most commonly been found in the jejunum (the terminal section of the intestinal tract) at the sites of Payer's patches (Amemori et al., 2004). However, *MAP* has also been detected outside the intestinal tract: in parenchymatous organs (liver, spleen, kidney, mammary gland and the reproductive tract), and in cow's milk and bull semen (Pavlik et al., 2000b; Ayele et al., 2004, 2005; Brady et al., 2008).

Due to the present intensive deer farming (particularly red deer and fallow deer) as well as cattle, the question arises as to whether *MAP* may also spread to the majority of their tissues. From the point of view of food safety it is necessary to answer this question of the potential occurrence of *MAP* in tissues other than the intestinal tract. Studies published so far dealing with the culture detection of disseminated *MAP* infection in wild ruminants have been performed under experimental conditions (Williams et al., 1983), or only isolated cases have been described (Von Deutz et al., 2003). In other studies, the identification of *MAP* has been made only in either the gastrointestinal tract (Jessup et al., 1981; Chiodini and Van Kruiningen, 1983; DeLisle et al., 1993; Power et al., 1993; Pacetti et al., 1994; Nebbia et al., 2000; Marco et al., 2002) or in different lymph nodes (Temple et al., 1979; DeLisle et al., 2003).

The epidemiology of *MAP* infection in domestic ruminants, farmed deer and free living ruminants has been studied by the standardized IS*900* Restriction Fragment Length Polymorphism (RFLP) method (Pavlik et al., 1999); that is, the method has been used to investigate *MAP* transmission from cattle to rabbits (*Oryctolagus cuniculus*; Greig et al., 1999), hares (*Lepus europaeus*; Machackova et al., 2004), brown bears (*Ursus arctos*; Kopecna et al., 2006) and different species of free living ruminants (Pavlik et al., 2000a; Machackova et al., 2004; Machackova-Kopecna et al., 2005). This method has also been used for the investigation of *MAP* occurrence in the environment and its transmission among invertebrate animals (Fischer et al., 2003, 2004, 2005; Machackova et al., 2004)

The first purpose of the present study was the investigation of *MAP* distribution by cultivation of faeces, serology and RFLP analysis in one red deer farm during a three year period. The second purpose was to study *MAP* distribution in the respective organs of ten euthanized animals and to assess the possible route of transmission in one herd of farmed red deer using the standardized IS*900* RFLP analysis.

MATERIAL AND METHODS

Epizootiological history of the studied deer farm

The deer farm, located in the midlands of the Czech Republic, was established by the purchase of red deer and fallow deer from one game park and seven deer farms. Nine years later, several young hinds from an imported deer herd in Scotland were purchased. Apart from this, since the establishment of the farm several purchases, amounting to a small number of red deer, were made from a farm located in the Czech Republic. The numbers of deer at this time varied from 50 to 100 animals and were kept in an area covering less than 5 ha. Two years later, after the purchase of the animals from Scotland, during a survey of paratuberculosis, *MAP* was isolated from the gastrointestinal tract of one emaciated hind on this farm. The *MAP* isolate from the hind was further typed by the IS*900* RFLP method as RFLP type B-C16 as reported by a previous study (Pavlik et al., 2000a). The present study was carried out during the three years following this. The number of animals on the farm varied from 55 to 71 during these three years.

Relationship between the infection status of mothers and their progeny

Anamnestic data from seven mothers (designated as A-G) of nine slaughtered progenees were obtained from the farmer and from our database of the culture results. Mothers originally came from the farm "PM" with a history of *MAP* with the profile B-C1 and farm "KT" with a history of *MAP* of profile M-C16 (Pavlik et al., 1995, 2000a).

Examined biological material

In the monitored three-year period a total of 242 faecal samples and 272 blood sera samples were examined by culture and serologically using the complement fixation test (CFT), respectively, the samples were taken at four examinations over the three years from all animals older than 12 months (Table 1).

In the last year of the investigation, based on clinical signs, serology and faecal culture, ten animals (4 females and 6 males) were euthanized. During the *post mortem* examination, 195 tissue samples were collected for laboratory examination from each slaughtered red deer. Milk from one hind was collected into a sterile tube by the massage of a cleaned and disinfected mammary gland. After incision of the amniotic sac of another hind with a sterile scalpel, amniotic fluid was collected into a sterile tube (Table 3).

Laboratory examinations

Serum. After delivery to the laboratory, blood samples were centrifuged and sera were stored at –20°C before examination for the presence of antibodies against *MAP* which was performed within three weeks. All sera samples were examined using the CFT and sera from euthanized animals were also tested by the immune diffusion test (IDT; both produced by Bioveta, Ivanovice na Hane, Czech Republic) following the manufacturer's instructions.

Necropsy material. Organs were stored at –20°C for up to two months before culture examination. After gross examination, tissue samples were examined microscopically after staining the smears according to the Ziehl-Neelsen (Z-N) method for the detection of acid-fast rods (AFR). Each tissue sample (about 1 g) was homogenised in a stomacher (Kleinfeld Labortechnik GmbH, Gehrden, Germany) and decontaminated in 0.75% HPC (Hexadecyl Pyridinium Chloride: N-cetylpyridinium chloride monohydrate, No. 102340, Merck, USA) for 72 h (Pavlik et al., 2000b). The sediment (200 µl) of each decontaminated sample was cultured on three slopes of different Herrold's egg yolk media (HEYM) and incubated at 37°C for 12 months according to a previously published procedure (Machackova et al., 2004).

Faecal samples. Faeces were stored at –20°C for up to two weeks before culture examination. Approximately 1 g of sample was transferred into a 50 ml flask containing 30 ml of sterile distilled water and agitated in a horizontal shaker for 30 min. Subsequently, the bottle was left undisturbed for a further 30 min so that the large particles might settle; 5 ml of the supernatant was transferred into a 50 ml bottle containing 25 ml of 0.9% HPC solution (Whipple et al., 1991; Pavlik et al., 2000b) and decontaminated for three days at room temperature. 200 µl of the sediment were then inoculated on the different media as described above.

Milk and amniotic fluid. Milk and amniotic fluid samples were stored at +4°C for one day after collection until they were examined by culture. The samples were centrifuged for 15 min at 2 500 rpm (650 × g) and after decantation of the supernatant, 5 ml of 0.75% HPC was added to the pellet. The sample was left undisturbed for 4 h at room temperature and centrifuged again for 15 min at 2 500 rpm (650 × g). The pellet was diluted with 1 ml of sterile distilled water and inoculated as described above.

Isolate identification and differentiation

Identification. All mycobacterial isolates from faeces, tissues, milk, and amniotic fluid were identified by the Mycobactin J dependence test: the subculture of each isolate on three HEYM with Mycobactin J and one HEYM without Mycobactin J according to Pavlik et al. (2000b), and subsequently by the IS*900* polymerase chain reaction (PCR; Bartos et al., 2006; Moravkova et al., 2008a).

Infection intensity in faecal and tissue samples. The results of faecal and organ cultures were assessed according to a previously published method (Pavlik et al., 2000b). Colony forming unit (CFU) counts were determined as the mean CFU of the three HEYM media used for the culture of one sample. Faecal samples with the growth of less than 10 CFU were assessed as slightly positive, 11 to 100 CFU as moderately positive and more than 101 CFU as highly positive. The intensity of infection was determined by calculating the mean CFU of all positive cultures. The intensity of tissue infection by *MAP* was evaluated using an identical scale as for the intensity of the shedding of *MAP* through faeces.

Differentiation. A total of 99 *MAP* isolates (53 from faeces and 46 from tissue) were differentiated by the standardised IS*900* RFLP method (Pavlik et

al., 1999). Digestion of DNA was performed using the restriction endonucleases *Bst*EII and *Pst*I. The group of isolates comprised isolates from all samples of tissue, faeces, milk and amniotic fluid of the culled ten red deer, from faeces within the global search culture.

Statistical analysis

The Chi-square (χ^2 test) and Fisher tests were performed to analyse the results (GraphPad Software, Inc., USA).

RESULTS

Serological examinations on the herd

During the monitored period serological examinations were repeated four times using the CFT; the production of antibodies for *MAP* increased from 0 in the first year to 7.7% (positive) and 0 to 88.5% (dubious) in the third year of the study (Table 1). The responses were mostly observed at the last performed examination in the third year of investigation, when positive and dubious responses were detected in 75 animals (96.2%; Table 1). A statistically significance increase ($P < 0.01$) in infected animals between the second and third year was seen.

Culture examinations of faeces from the herd

The initial global culture examination of faeces from 28 animals older than 12 months was negative for *MAP*. In the three subsequent examinations of 68, 78, and 68 animals, the following positivity was found: 5.9%, 34.6%, and 36.8%, respectively. A statistically significance increase ($P < 0.01$) in infected animals between the first and second, and the second and third year was seen. Fifty-two of the 56 *MAP* isolates obtained from animals and paddock isolate were examined by the RFLP method; these were all of the RFLP type B-C1 (Table 1).

Examination of ten euthanized animals

Clinical and serological examinations. According to the data recorded by the farmers and the vet-

Table 1. Serological and faecal culture examinations of *Mycobacterium avium* subsp. *paratuberculosis* from red deer (*Cervus elaphus*) older than 12 months of age

Examinations		Serum[a]			Faeces[b]			RFLP typing[c]	
year	month	No.	+ (%)	± (%)	No.	+	%	No.	B-C1
1st	May	NT			28[d]	0	0	0	0
	August	55	0 (0)	0 (0)	NT			NT	
2nd	April	71	2 (2.8)	0 (0)	NT			1[e]	1[e]
	September	68	2 (2.9)	6 (8.8)	68	4	5.9	4	4
3rd	April	78	6 (7.7)	69 (88.5)	78	27	34.6	23	23
	September	NT			68	25	36.8	25	25
Total No.		272	10 (3.7)	75 (27.6)	242	56	23.1	53	53

[a]complement fixation test for the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* was used: + = positive (reaction for +++ in the dilution 1 : 10 and higher dilutions), ± = dubious (reactions up to ++ in the dilution 1 : 10)

[b]culture examination according to a previously described method (Pavlik et al., 2000b) was carried out

[c]isolates from faeces, *M. a. paratuberculosis* isolates were analysed with a standardised IS*900* RFLP method described previously (Pavlik et al., 1999)

[d]only hinds were examined

[e]one *M. a. paratuberculosis* isolate was obtained from two soil samples collected in the paddock and examined as faecal samples

NT = not tested

erinarian who collected faecal samples, no signs of chronic diarrhoea or emaciation were observed in any of the animals during the first two years of the monitored period. In the third year, signs of chronic diarrhoea were observed in four out of ten euthanized animals (Table 2). Faeces in the rectum were watery, shapeless and greenish in colour; the skin around the tail was soiled with them.

Extreme emaciation was not observed in any of the animals. Positive or dubious responses were detected in nine and three animals by the CFT and IDT, respectively. All clinically suspect animals were found positive by examination with the CFT and a positive response was also observed in two of them by the IDT (Table 2).

Faecal cultures. Culture results were negative in two animals that were also negative by faecal culture in a previous examination in the second year of the study. Faecal culture was positive in eight remaining animals: mild shedding was found in three animals (up to 10 CFU), moderate shedding in another three animals (11 to 100 CFU) and massive shedding (more than 101 CFU) in the remaining two animals. All eight *MAP* isolates were of RFLP type B-C1 (Table 2).

Gross examinations. Gross lesions of intestinal mucosa (gyrification and blood vessel injection) and intestinal lymph nodes (enlarged and oedematous) were observed in all red deer stags and two hinds (Table 2). Tuberculoid granulomatous lesions were observed in the mesenteric lymph nodes of one red deer stag; particularly distinct lesions were localized in jejunal lymph nodes adjacent to the middle part of the jejunum (Figure 1).

Microscopy of homogenised samples prior to culture examination. AFR were detected in 23 tissue samples from only three stags. In the 2-year-old red deer, Nos. 2 and 3, AFR were detected in imprint specimens of intestinal mucosa from the entire digestive tract. AFR were also detected in the liver, hepatic, and mediastinal lymph nodes of stag No. 2. In the one-year-old red deer stag No. 6 AFR were occasionally detected and only in the duodenal lymph node (Table 3).

MAP distribution in different organs determined by the culture of tissue, milk and amniotic fluid

MAP was detected in the organs of all 10 examined animals. *MAP* was isolated from the intestinal tract and pulmonary (tracheobronchial or mediastinal) lymph nodes of all studied animals. Also the presence of *MAP* in kidneys, liver or spleen was noteworthy. *MAP* was also isolated from female organs: mammary glands (hinds Nos. 9 and 10), supramammary lymph node (hind No. 9) and uterus (hind No. 8), and from male organs: testes from red deer stags Nos. 1 and 2. *MAP* was likewise isolated from the amniotic fluid and milk of the 2-year old hind No. 9 and the 5-year old hind No. 10, respectively (Table 3).

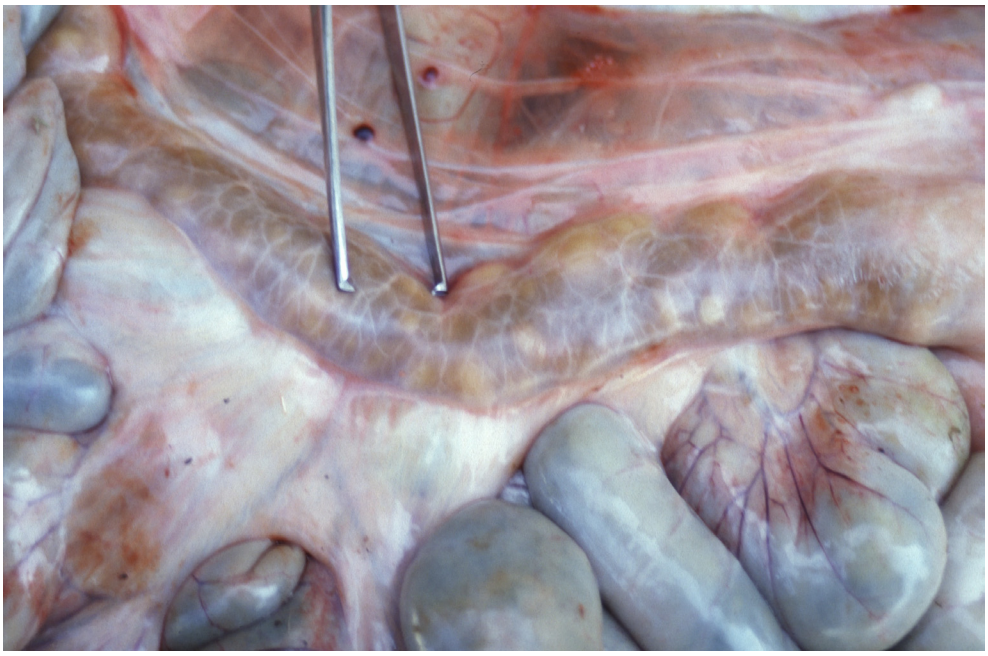

Figure 1. Enlarged jejunal lymph nodes with granulomas

Table 2. The results of clinical and laboratory examinations of 10 euthanized red deer (*Cervus elaphus*)

Animals		2nd year (April, September)				3rd year (April)				*Post mortem* examination in the 3rd year (April)					
				faeces				faeces		tissue				milk/amnion	
ID	gender (years)	clin. sign[a]	serol.[b] CFT/IDT	cult.[c]	RFLP	clin. sign[a]	serol.[b] CFT/IDT	cult.[c]	RFLP	P[d]	M[e]	cult.[c]	RFLP	cult.[c]	RFLP
1	stag (2)	–	±/NT	–		+	+/–	–		+[f]	–	+(30)	B-C1		
2	stag (2)	–	+/NT	+(20)	B-C1	+	+/+	+(500)	B-C1	+	+	+(250)	B-C1/C5		
3	stag (2)	–	+/NT	+(100)	B-C1	+	+/+	+(500)	B-C1	+	+	+(150)	B-C1/C5		
4	stag (2)	–	±/NT	–		+	+/–	+(100)	B-C1	+	–	+(30)	B-C1		
5	stag (1)	–	NT/NT	NT		–	±/–	+(10)	B-C1	+	–	+(50)	B-C1		
6	stag (1)	–	NT/NT	NT		–	±/–	+(50)	B-C1	+	+	+(100)	B-C1/C5		
7	hind (2)	–	±/NT	–		–	+/–	–		–	–	+(5)	B-C1	NT/NT	
8	hind (2)	–	±/NT	+(50)	B-C1	–	–/+	+(2)	B-C1	+	–	+(60)	B-C1/C16	NT/NT	
9	hind (2)	–	–/NT	+(1)	B-C1	–	±/–	+(1)	B-C1	–	–	+(60)	B-C1/C5	NT/+(1)	NT/B-C1
10	hind (5)	–	±/NT	–		–	+/–	+(20)	B-C1	+	–	+(20)	B-C1	+(2)/NT	B-C1/NT

[a]enteritis: + present, – not observed

[b]CFT = complement fixation test: + = positive (reaction for +++ in the dilution 1 : 10 and higher dilutions), ± = dubious (reactions up to ++ in the dilution 1 : 10), IDT = immunodiffusion test: + = positive (strong precipitation line connected with the positive control line), ± = dubious (weak precipitation line), – = negative (precipitation line was not observed)

[c]cult. = culture examination: – = negative, + = positive (number of colony forming units)

[d]P = gross findings: + = gyrification of mucosa and/or enlargement of lymph nodes, – = described gross lesions not observed

[e]M = microscopy after Ziehl-Neelsen staining: + = acid-fast rods (AFR) detected in clumps, – = AFR not detected

[f]tuberculoid granulomatous lesions were found in the mesenteric lymph nodes of this stag

NT = not tested

ID = identification No. of animal

Table 3. Distribution of different RFLP types of *Mycobacterium avium* subsp. *paratuberculosis* in red deer

Examined samples[a]	Total No.	Positive		No. of stag[c]						No. of hind[c]			
		Z-N[b]	culture	1	2	3	4	5	6	7	8	9	10
Ruminal LN	10	1	8	0	**1**[A]	1[A]	1[A]	0	1	1[A]	1	1[A]	1
Duodenal M	10	2	10	1	**1**	1	1	1	**1**	1	1	1	1
Duodenal LN	10	2	10	1	**1**	**1**	1	1	1	1	1	1	1[A]
Jejunal M	10	2	10	1	**1**	**1**	1	1	1	1	1	1	1[A]
Jejunal LN	10	2	10	1	**1**[A]	**1**	1	1	1	1	1	1[B]	1
Ileal M	10	2	10	1	**1**	**1**	1[A]	1[A]	1[B]	1	1[C]	1	1
Ileal LN	10	2	9	1	**1**	**1**	1	1	1	0	1	1	1
Ileocaecal valve M	10	2	10	1	**1**	**1**	1	1	1	1	1	1	1
Ileocaecal LN	10	2	10	1[A]	**1**	**1**[B]	1	1	1	1	1[C]	1	1
Caecal M	10	2	10	1	**1**	**1**[B]	1	1	1	1	1	1[A]	1
Rectal M	10	1	8	1	**1**	1	1	1	1	1	0	1	0
Faeces	10	0	8	0	1[A]	1[A]	1[A]	1[A]	1[A]	0	1[A]	1[A]	1[A]
Submandibular LN	10	0	8	1	1[B]	0	1[A]	1[A]	1	0	1	1	1
Tracheobronchial LN	10	0	9	1	1[A]	1	1	1	1	1[A]	1[A]	0	1
Mediastinal LN	10	1	9	1[A]	**1**	1[A]	1	1[A]	1[A]	0	1	1	1[A]
Liver LN	10	1	7	1	**1**	1	0	1	1	1[A]	1[A]	0	0
Liver tissue	10	1	7	1	**1**	0	1[A]	1	1[A]	0	1	0	1[A]
Spleen tissue	10	0	8	1	1	1[A]	1	1[A]	1	0	1	0	1[A]
Kidney	10	0	9	1[A]	1	1	1	1	1	1[A]	1	0	1
Mammary gland	4	0	2							0	0	1[A]	1[A]
Supramammary LN	1	0	1							NA	NA	1	NA
Milk	1	0	1							NA	NA	NA	1[A]
Uterus	2	0	1							NA	1[A]	0	NA
Amnion fluid	1	0	1							NA	NA	1[A]	NA
Testicles	6	0	2	1[A]	1[A]	0	0	0	0				
Total	205	23	178										
%	100	11.2	86.8										

[a]LN = lymph node, M = mucosa; [b]after Ziehl-Neelsen staining acid-fast rods were detected in clumps in the examined sample (in bold); [c]NA = not available; [d]randomly selected isolates were RFLP typed: [A]B–C1 RFLP type, [B]B-C5 RFLP type, [C]B-C16 RFLP type

RFLP analysis of tissue and faecal *MAP* isolates from ten euthanized animals

Three RFLP types B-C1, B-C5 and B-C16 were revealed by the examination of 54 randomly selected *MAP* isolates from a total of 178. RFLP type B-C1 (n = 47) was the most frequently observed and was much more common than RFLP types B-C5 (n = 5) and B-C16 (n = 2; Table 3). Mixed infection caused by isolates of RFLP types B-C1 and B-C5 was detected in four animals, Nos. 2, 3, 6 and 9, and a mixed infection caused by isolates of RFLP types B-C1 and B-C16 was recorded in animal No. 8. Four isolates of RFLP type B-C5 and both isolates of RFLP type B-C16 were detected in the intestinal tract, one isolate of RFLP type B-C5 was detected

Table 4. Infection statuses of mothers and their progeny

Mother[a]								Progeny				
1st and 2nd years of the study				3rd year of the study				3rd year of the study				
ID origin[a]	clinical signs[b]	CFT[c]	culture[d] F/T[e]	clinical signs[a]	CFT[c]	faeces		gender ID[f]	faeces		tissue	
						C[d]	RFLP		C[d]	RFLP	C[d]	RFLP
A-PM	–	–	–/NT	–	±	–		stag 1	–		+	B-C1
								stag 5	+	B-C1	+	B-C1
B-KT	–	–	–/NT	–	±	+	B-C1	stag 2	+	B-C1	+	B-C1, B-C5
C-PM	–	–	–/NT	–	±	+	B-C1	stag 3	+	B-C1	+	B-C1, B-C5
D-KT	–	–	–/NT	–	±	–		stag 4	+	B-C1	+	B-C1
E-KT	–	–	–/NT	–	±	–		hind 7	–		+	B-C1
F-KT	–	–	–/+ B-C16[g]	NT	NT	NT	NT	hind 8	+	B-C1	+	B-C1, B-C16
G-KT[h]	–	±	–/NT	–	+	+	B-C1	hind 9	+	B-C1	+	B-C1, B-C5

[a]seven mothers (designated as A-G) originated from studied herd PM, where *M. a. paratuberculosis* of RFLP type B-C1 was diagnosed in 1997, and from herd KT with imported red deer from Scotland in 1992 with diagnosed *M. a. paratuberculosis* of RFLP type M-C16 in 1997

[b]enteritis: – = not observed, ± = intermittent enteritis was observed, + = chronic enteritis was present

[c]CFT = complement fixation test: + = positive (reaction for +++ in the dilution 1 : 10 and higher dilutions), ± = dubious (reactions up to ++ in the dilution 1 : 10).

[d]C = culture examination and RFLP analysis of *M. a. paratuberculosis* isolates

[e]F = faecal culture/T = tissue culture and RFLP analysis of *M. a. paratuberculosis* isolates

[f]genders studied in detail in this paper (for more details see Tables 2 and 3)

[g]the hind died in September in the 1st year of the study and *M. a. paratuberculosis* was isolated from the liver and mesenteric lymph nodes.

[h]mother G of hind No. 9 was identical with hind No. 10 in our study

ID = Identification of animal; NT = not tested

in a submandibular lymph node, and RFLP type B-C1 was found in all types of tissue samples as well as in faeces, milk and amniotic fluid.

Relationship between the infection status of mothers and their progeny

The infection status of seven mothers of ten infected euthanized progenies was very similar. In the third year of the study *MAP* was detected in the faeces of three mothers. The RFLP profile of these isolates was B-C1. One mother died in the first year of the study and *MAP* with the RFLP profile B-C16 was isolated from her tissues. The CFT test revealed a positive result in only one mother while the remainder of the mothers gave a dubious reaction to this test (Table 4).

DISCUSSION

Paratuberculosis is mainly a problem of domestic ruminants, whose symptoms include diarrhoea, weight loss and a reduction in milk production. Despite the fact that since the 1980s paratuberculosis has also emerged as a problem on deer farms in many countries, e.g., UK, New Zealand, Czech Republic and USA (DeLisle et al., 1993; Fawcett et al., 1995; Manning et al., 1998; Pavlik et al., 2000a; Machackova et al., 2004), information about this disease in deer is still missing or is incomplete. In the present survey we studied the spread of *MAP* in one red deer farm by faecal culture, serology and RFLP analysis during the three year period. To better understand the route of transmission we also studied the distribution of *MAP* in ten animals that were assessed as positive according to serology or faecal culture.

The application of a suitable method for the diagnosis of *MAP* infection in different animal species is still under the discussion. There is as yet no intravital diagnostic method that is able to reveal all infected animals. In accordance with the literature (Power et al., 1993; Fawcett et al., 1995; Marco et al., 2002), a low sensitivity of both serology tests in red deer was observed in the present study (Tables 1, 2 and 4). The best results of the CFT were observed in clinically affected animals in comparison with subclinical cases which gave usually dubious reactions. According to our study and other authors (Quist et al., 2002; Davidson et al., 2004), the IDT method was not found to be suitable for the detection of *MAP* infection in deer. The IDT assay positive results gave for only three animals from the ten infected compared with the CFT which produced positive results in five out of ten animals and three dubious results (Table 2). The drawbacks of IDT and CFT have also been noted in the *MAP* diagnosis in cattle and sheep (Williams et al., 1983; Ayele et al., 2001; Kohler et al., 2008) and in farmed deer (Mackintosh et al., 2004).

Regardless of the fact that faecal cultivation did not reveal all infected animals (based on the results of the cultivation of tissues), according to our study examination by faecal culture was more successful at revealing infected animals (Tables 2 and 4) than serological methods. Furthermore, faecal cultivation detects animals that are shedding *MAP* in their faeces, which poses the highest risk factor for spreading the infection in the herd. The failure to detect *MAP* in the faeces of two red deer, from which *MAP* was isolated from tissue samples (Table 2), has also been described for cattle (Pavlik et al., 2000c; Whittington et al., 2004) and for Key deer (Quist et al., 2002). The negative result in faecal culture might have been caused by the irregular and/or low shedding of *MAP* in faeces; the insufficient sensitivity of the sedimentation culture method or devitalisation effect of HPC could be other reasons (Pavlik et al., 2000b). Therefore, repeated faecal culture at intervals of several weeks or months or quantitative real time PCR method is recommended.

The spread of *MAP* on the farm and the development of clinical signs of infection may be influenced by different stress factors. In our case, a high density of animals (16 or more red deer per 1 ha depending on the extent of pastures) and the very bad conditions on the farm likely facilitated a fast spread of infection among a majority of animals. Clinical signs such as diarrhoea and weight loss usually appear in deer aged 8 to 15-months (Mackintosh et al., 2004). This was confirmed in our study; clinical signs were seen in two-year old animals (Table 2).

Gross lesions (such as greatly enlarged and oedematous jejunal and ileocaecal lymph nodes) and mild to moderate intestinal thickening have been described for farmed red deer (Clarke, 1997; Mackintosh et al., 2004) and comparable findings were recorded in the present study. Moreover, we observed caseous lesions (creamy in colour) in the mesenteric lymph nodes of one stag which is also

occasionally described for red deer (DeLisle et al., 1993; Mackintosh et al., 2004). These lesions in deer lymph nodes may closely resemble those seen in *M. bovis* (Mackintosh et al., 2004) or *M. avium* subsp. *hominissuis* infected ruminants (Moravkova et al., 2008b).

AFR were revealed by microscopy in only three red deer in the present study, although *MAP* was isolated from most of the tissue samples of all red deer (Tables 2 and 3). In contrast, the majority of cases of tissues culture positivity for *MAP* were confirmed by AFR detection in tissue smears (Jessup et al., 1981; DeLisle et al., 1993; Power et al., 1993). The fact that tissues were AFR negative (even in the case of a red deer stag with tuberculoid lesions in mesenteric lymph nodes) can be explained by the long incubation time of the disease and the early stage in which it was in the majority of animals investigated in the present study (Tables 2 and 3).

Based on the experience obtained from paratuberculosis control in cattle (Pavlik et al., 2000c), it is possible to decrease the incidence of this disease by a timely culling of all animals in which *MAP* has been detected by faecal culture. It is also possible to further improve control by culling all progeny of infected mothers, because these are often infected with *MAP* from their mothers either *in utero* or after birth by ingestion of infected colostrum or milk, depending on the intensity of infection of the mothers (Sweeney et al., 1992b).

In this study, intestinal tract and adjacent lymph nodes were more commonly found to be *MAP* infected tissues in the euthanized red deer (Table 3) compared to the detection rates of *MAP* in parenchymatous organs or other lymph nodes ($P < 0.01$). Similar results were described for cattle (Pavlik et al., 2000b; Amemori et al., 2004) and experimentally infected deer (Williams et al., 1983). Comparable results for *MAP* isolation from extraintestinal tissues, obtained especially in cattle (Whitlock and Buergelt, 1996; Pavlik et al., 2000b; Ayele et al., 2004), have been recorded for free living ruminants, above all deer species (Williams et al., 1983; Manning et al., 1998; DeLisle et al., 2003; Machackova-Kopecna et al., 2005; Kopecna et al., 2008). It has been shown that the respiratory tract may be infected with *MAP* present in aerosol, particularly in herds with a high density of animals (Williams et al., 1983; Pavlik et al., 2000b; Machackova-Kopecna et al., 2005). This is also supported by the fact that *MAP* was isolated from tracheobronchial or mediastinal lymph nodes from all red deer in the present study (Table 3).

Apart from the transmission of *MAP* by ingestion and inhalation (mentioned above), another potential way of transmission is through the reproductive organs. The isolation of *MAP* from the testes, semen or foetus of cattle was described by (Larsen et al., 1981; Seitz et al., 1989; Sweeney et al., 1992b; Ayele et al., 2004; Glawischnig et al., 2004). Thompson et al. (2007) described an intra-uterine transmission rate of 78% in infected red deer. This is in agreement with our isolation of *MAP* from the reproductive organs of naturally infected red deer of both sexes (Table 3).

The isolation of *MAP* from milk, mammary glands and associated lymph nodes hints at a possible early transmission of *MAP* from mother to young deer which is generally described in cattle (Sweeney et al., 1992a; Slana et al., 2008).

In this study, when the vertical spread of infection was investigated, *MAP* was detected in the faeces of three clinically healthy mothers of four infected progeny, and in the organs of the mother of one young deer. Dubious reactions with the CFT assay for the detection of *MAP* antibodies was observed in six tested mothers (Table 4). The RFLP profile of these isolates from faeces was B-C1 and one isolate from the tissue of a dead hind was assigned the profile B-C16. If we take in account the hypothesis of intrauterine transmission, a mixed infection with *MAP* of two profiles B-C1/B-C5 and B-C1/B-C16 in five progeny could be explained in this way, since all infected animals in the herd shed *MAP* with the profile B-C1. Unfortunately, we were not able to test the tissue of the mothers of the six progeny and to so explain the B-C5 profile that was present in the progenies' tissues.

Another possible explanation for the isolation of two profiles from one animal could be genetic mutations of *MAP*. Only minor changes in RFLP profiles were observed and might have resulted from minute evolutionary changes within one population. Differences in RFLP profiles between RFLP types B-C1 and B-C5 are represented by only one band, corresponding to a simple replicative transposition of an IS*900* element in RFLP type B-C5. The difference between RFLP types B-C1 and B-C16 consists in the presence of one extra copy of IS*900* in B-C16 and a minor change in the length of one band in the range of hundreds of bp. It is not possible to unequivocally explain the origin of these changes; they might result from the transposition

of an IS*900* and from a parallel point mutation adjacent to another copy of IS*900* or another event (site-specific recombination) between two IS*900*. However, according to our experience, RFLP profiles in *MAP* are very stable and a change in RFLP profiles is not a very probable event, especially a change between profiles B-C1 and B-C16.

Despite the fact that we investigated a relatively closed group of animals we detected three different RFLP types of *MAP*: B-C1, B-C5 and B-C16. We also obtained similar results in other herds of farmed deer in the Czech Republic (Machackova et al., 2004; Machackova-Kopecna et al., 2005). This could be explained by the purchase, on a number of occasions, of a small number of red deer from a farm located in the Czech Republic. We assume that the source of the RFLP type B-C16 might have been animals imported from Scotland where this RFLP type occurs in deer and rabbits (Greig et al., 1999). The RFLP profile B-C1 that was isolated from the faeces of all infected animals and from the majority of tissues, is the most predominant profile in cattle and free living ruminants in the Czech Republic. On the basis of our previous monitoring of deer farms with mixed infections B-C1 and M-C16, where the profile M-C16 was gradually replaced with profile B-C1, it is supposed that the profile B-C1 is more virulent or more resistant to stress factors (Machackova et al., 2004).

The present study suggests a possible vertical and horizontal transmission of *MAP* in red deer which was examined by the distribution of *MAP* in different organs or tissues and by RFLP analysis of detected isolates (Table 4). The most probable way of transmission was the faecal-oral route, but transmission by the inhalation of infected aerosol or intra-uterine transmission cannot be ruled out. Similar results were observed by Judge et al. (2006) in rabbits in the UK. The isolation of *MAP* from many tissue types (Table 3) suggests various routes of transmission including highly persistent nature of *MAP* in livestock and the fact that paratuberculosis is difficult to control in infected herds.

Acknowledgements

The authors would like to thank doc. RNDr. Milan Bartos, Ph.D., Mgr. Petra Svastova, Ing. Zdenka Rozsypalova and Marcela Fisakova from the Veterinary Research Institute (Brno, Czech Republic), and MVDr. Michael Caras from the Veterinary and Pharmaceutical University (Brno, Czech Republic) for technical assistance. They also wish to thank the staff on the red deer farm for providing us with anamnestic data. Thanks are also due to Zdenka Gregorova and Anna Maslanova from VRI for finding all literature and Ing. Ludmila Faldikova from VRI for the assistance with the translation. Neysan Donnelly (Aberdeen University, United Kingdom) is acknowledged for grammatical correction of the manuscript.

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Received: 2008–07–08
Accepted after corrections: 2008–09–30

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