Mycobacterium avium subsp. *paratuberculosis* in a mouflon herd without clinical symptoms monitored using IS*900* real-time PCR: a case report

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ABSTRACT: The aim of this study was to monitor over two years a farmed mouflon herd for the presence and persistence of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) using an IS*900* real-time PCR method. This study followed the previous monitoring of the herd using a cultivation method which showed only a minimal infection load among the animals. Although no mouflon showed clinical symptoms, 35.7% and 80% of ewes were IS*900*-positive in 2008 and 2009, respectively. In seven out of 21 adult ewes, the presence of the IS*900* sequence was determined in 2008 as well as in 2009. Between the first and second sampling, twenty-three mouflon lambs born and kept with the ewes were examined. Almost one third of them (30.4%) were proven to have the *MAP* sequence in their faeces. Also, 75% environmental samples from the mouflon farm showed positivity. Infected animals without clinical symptoms which low sensitivity cultivation does not detect represent a source of infection for other animals. Therefore, real-time PCR has a crucial role in paratuberculosis control programs, especially in control of the disease by the culling of infected animals.

Keywords: *MAP*; Johne's disease; faeces; environment; qPCR

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is the causal agent of paratuberculosis (PTB, Johne's disease), mainly found in domestic ruminants. Ruminants living in the wild or kept in farms, e.g. red deer, fallow deer and roe deer have been described to be quite often infected by *MAP* (Godfroid et al., 2000; Marco et al., 2002; Deutz et al., 2003, 2005; Machackova et al., 2004). The Alpine ibex (Ferroglio et al., 2000), tule elks (Manning et al., 2003) or alpacas (Ridge et al., 1995) belong to other ruminants in which *MAP* has been found. Apart from ruminants, *MAP* has also been described in various non-ruminants (Greig et al., 1999; Beard et al., 2001; Anderson et al., 2007). However, regardless of the species, infected animals with or without clinical symptoms excrete *MAP* through their faeces into the environment and thus pose a risk to grazing livestock and other animals (Beard et al., 2001).

In the Czech Republic, PTB in free living ruminants (a game preserve) was first described in 1976 in a red deer (Krucky, 1981). Since then, the disease has been observed in all breeds of wild ruminants. Between 1995 and 1998, forty-eight mouflons from game preserves and from the wild were examined for the presence of *MAP* using cultivation. Two mouflons (4.2%) were diagnosed as positive (Pavlik et al., 2000). Subsequently, a more extensive study on wild ruminants in the Czech Republic was carried out using cultivation from intestine, corresponding lymph nodes and faecal samples. Sixteen mouflons (3.8%) were *MAP*-positive, although nine did not show any clinical symptoms of the disease (Machackova et al., 2004). From 2002 to 2007, *MAP*

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was found in 3.2% of the 866 mouflons examined in the Czech Republic (Kopecna et al., 2008). All above mentioned studies were performed using cultivation and histological examination. In Austria, evidence of *MAP* in mouflons was performed using cultivation and conventional PCR (Deutz et al., 2005) and in Spain using the serological examination of blood (Lopez-Olvera et al., 2009). However, no previous study to date has quantified the amount of *MAP* in mouflon faeces, particularly in the faeces of animals without clinical symptoms.

The aim of this study was to continue the long term monitoring of *MAP* in a farmed mouflon herd. The herd was first examined by culture in 2002 after the appearance of clinical signs (Kopecna et al., 2008). Between 2002 and 2003, five animals died from paratuberculosis. After the culling of the clinically ill animals, clinical signs disappeared and further *MAP* cultivation was negative in all animals. In 2006, one clinically ill ewe was euthanized and a massive *MAP* infection was diagnosed in its gastrointestinal tract. However, the agent was not isolated even after twelve months of cultivation. IS*1311* PRA-PCR (Marsh et al., 1999) applied to a DNA sample from the tissue of this mouflon revealed that a *MAP* "sheep" strain (which did not grow *in vitro*) was present (Kopecna et al., 2008). Cultivation of faecal samples from all other adult animals was negative in 2006, as well as in subsequent years (data not published).

Because of our goal of verifying the real health status of animals without clinical symptoms, real-time quantitative PCR (IS*900* qPCR) was used in this study. Moreover, the use of qPCR allowed us to ascertain with a higher degree of certainty the amounts of *MAP* in animal faeces. In order to examine possible sources of *MAP* persistence in the farm, environmental samples were also analysed. The flock consisted of mouflon ewes and their lambs, and the influence of an *MAP*-contaminated environment on the lambs from birth could be observed.

Case description

Mouflons farm. The mouflon farm located in the Czech Republic has an area of seven hectares. In 2008, the flock of ewes consisted of 32 animals and this number increased to 55 animals in 2009 due to 23 newly-born lambs. The flock of lambs consisted of lamb rams aged up to six months and lamb ewes. New animals (generally adult rams only) were purchased exclusively from other farms (a regular exchange occurs in annual intervals before the rut season); no wild animals were imported into the

Table 1. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in the mouflon farm environmental samples using IS*900* quantitative real-time PCR

Sample No.	Localisation	Type of the sample	Number of *MAP* cells per gram of sample
1	feeding place	soil from path to the feeding place	3.52×10^4
2		disinfected soil	3.57×10^3
3		remains of feed	2.25×10^3
4		body scratcher for animals	2.24×10^3
5		remains of faeces	2.13×10^3
6		water sediment from watering place	2.86×10^2
7		spider's web	1.58×10^2
8		spider's web	6.99×10^1
9	natural well	algae	0
10		organic remains	0
11	catch-drain system	biofilm from the drainpipe	0
12		sediment close to the absorption pit	2.33×10^3
13		organic sediment from the distribution tile	9.82×10^2
14	others	soil with urine	5.41×10^3
15		soil from the path out of the game preserve	6.10×10^1
16		feed without previous contact with animals	0

herd. The farm was not in contact with any other deer species or farm animals. The farm paid particular attention to the welfare of its animals, and to mechanical cleaning and disinfection of sites and equipment.

Sample collection and examination. At the beginning of October 2008 and 2009 mouflon ewes and lambs were captured and rectal samples of faeces were collected (using individual disposable gloves for each animal). In 2008, 28 out of 32 captured animals were examined as four animals did not have enough faeces in their rectum. For the same reason, 25 out of 32 ewes were examined in 2009; all 23 lambs were examined without exception. In spring 2009, environmental samples were collected into sterile plastic bags using sterile spatulae (Table 1). After homogenisation, 250 and 500 mg of environmental and faecal samples, respectively, were used for isolation of DNA. DNA from faecal samples was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Germany); DNA from environmental samples using the PowerSoil DNA Isolation kit (MoBio, USA) according to the manufacturer's recommendations. All DNA samples were examined using IS*900* qPCR and the absolute number of *MAP* cells per 1 g of faeces was calculated as described previously (Slana et al., 2008). Until the examination was completed, all samples were kept at –80 °C. qPCRs were carried out in duplicate for each sample.

Statistical analysis. The rates of positive animals were compared by Fisher's exact test (GraphPad Prism 5.02, GraphPad, Inc., SCA, USA).

RESULTS

The IS*900* sequence was found in ten (35.7%) and 20 (80%) ewes in 2008 and 2009, respectively. From the 21 adult animals with a sufficient quantity of faeces in both 2008 and 2009, two ewes (9.5%) were found to be negative and seven (33.3%) positive in both years. The concentration of *MAP* cells in the faeces of ewes ranged between 1.00×10^0 and 1.89×10^7 in both years (Table 2). An examination of lamb faeces revealed the presence of the IS*900* sequence in seven cases (30.4%). Positivity among lambs ranged between 2.92×10^2 and 1.25×10^3 *MAP* cells (Table 2; negative lambs were not included).

With regard to environmental samples, twelve were *MAP* positive (75%; Table 1).

DISCUSSION AND CONCLUSIONS

The results of IS*900* qPCR showed that paratuberculosis infection had persisted over the course of years within the mouflon herd without the occurrence of clinical signs and positive culture since 2006. Moreover, the increase in positive animals in the second year of qPCR monitoring compared to the first year was evident and statistically significant (Table 2; $P < 0.01$; P is P-value for Fisher's Exact Test). Two explanations for such high variations are possible: (i) the occurrence of intermittent shedding (Palmer et al., 2007) in animals during the sample collection in 2008 or (ii) more frequent and long-time exposure of animals to the agents in 2009 compared to 2008. In the present study, the concentration of *MAP* ranged from 10^1 to 10^3 in most adults. However, in one animal 1.89×10^7 *MAP* cells were found, although it did not show clinical signs of the disease (Table 2). This animal was probably the most important source of *MAP* infection for other ewes and lambs.

Compared to adults, the occurrence of the clinical disease in young animals is more frequent and is accompanied by a rapid loss of body condition (Frolich et al., 2002; Deutz et al., 2003). No ewe or lamb tested in this study showed clinical signs of paratuberculosis even though *MAP* was detected in adults as well as young animals (Table 2). Surprisingly, in the faeces of lambs, between 10^2 to 10^3 *MAP* cells were detected (Table 2) which could indicate infected animals rather than passive shedding only. Passive shedding was demonstrated in calves stabled with highly shedding cows. After removal of shedding cows, *MAP* excretion became less frequent in the calves (Van Roermund et al., 2007). Intrauterine transmission cannot be excluded in such cases as Whittington and Windsor (2009) described intrauterine transmission in 9% of foetuses from sub-clinically infected cows.

In various field studies using different methods (Whittington et al., 2003; Berghaus et al., 2006), *MAP* was detected in environmental samples collected from animal farms. In the present study, IS*900* qPCR detected *MAP* in 75% of the environmental samples, even though the owner of the mouflon farm followed strict hygienic measures (mechanical cleaning, permanent liming in the surrounding of feeding places, etc.). In compliance with Whittington et al. (2004), *MAP* (3.57×10^3) was found in a sample of limed soil (Table 1). However, as viable cells were not scored in this

Table 2. *Mycobacterium avium* subsp. *paratuberculosis* detection in faeces of mouflon lambs and ewes using IS*900* quantitative real-time PCR (IS*900* qPCR)

No.	Age of animals (2009)	Number of *MAP* cells per g of faeces (based on IS*900* qPCR)	
		2008	2009
1	lambs (< 1 year)		7.69×10^2
2			6.95×10^2
3[a]			4.90×10^2
4[a]			6.58×10^2
5[a]			2.92×10^2
6[a]			8.46×10^2
7[a]			1.25×10^3
8	ewes (from 1.5 to 9.5 years)	0	0
9		0	0
10		1.00×10^0	2.76×10^2
11		2.41×10^2	1.89×10^7
12		2.68×10^2	2.00×10^2
13		1.83×10^2	2.09×10^1
14		6.50×10^2	4.33×10^1
15		4.94×10^2	8.51×10^2
16		7.21×10^4	2.76×10^2
17		0	2.37×10^1
18		0	8.14×10^1
19		0	7.31×10^1
20		0	3.12×10^2
21		0	7.66×10^2
22		0	7.27×10^2
23		0	4.15×10^2
24		0	6.10×10^2
25		0	2.74×10^2
26		0	1.75×10^3
27		0	2.30×10^3
28		2.64×10^3	0
29		NA	0
30		NA	0
31		NA	1.40×10^2
32		NA	4.06×10^2
33		0	NA
34		0	NA
35		0	NA
36		0	NA
37		0	NA
38		1.89×10^2	NA
39		3.99×10^2	NA

Lambs with negative results (16 lambs) are not included in the table
NA = samples not available (not enough faeces)
[a]males

study, we cannot conclude that *MAP* survived the liming treatment. Even though PCR does not allow a distinction between live and dead cells, its advantage lies in the fact that it can demonstrate the presence of *MAP* with significantly higher sensitivity than culture.

In conclusion, this study showed a continuously proceeding *MAP* infection in a farmed mouflon herd, in which the presence of *MAP* was not demonstrated using culture methods since 2006. IS*900* qPCR analysis in two consecutive years (2008 and 2009) showed the presence of *MAP* not only in the faeces of adult ewes, but also in lambs born between the first and second sample collection. Additionally, examination of the farm environment showed a high level of contamination which represents a significant source of infection for the animals. From these results we conclude that qPCR has an indispensable role in paratuberculosis control programmes and its implementation along with cultivation should be recommended. In addition, as qPCR targets nucleic acid, false negative results due to the presence of non-growing "sheep" strains can be avoided.

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