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Original article

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Slovakian wildlife

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of infectious enteritis called paratuberculosis that has a high economic impact on the worldwide livestock production. A central important question arises: Can wildlife animals serve as a reservoir for transmission of MAP to domestic ruminants? With this in mind, we devised a study to detect MAP in various Slovakian wildlife species found in the areas where paratuberculosis had been documented in domestic ruminants. The samples of parenchymatous organs (intestines, ileocecal valve and mesenteric lymphatic nodes) from 83 wildlife animals representing 13 species, including 7 herbivorous, 5 carnivorous and 1 omnivorous species were collected during a four-year period. The clinical and pathological examinations failed to demonstrate any manifestations of paratuberculosis in any of the wildlife samples. The detection of MAP was done by widely used tests, i.e. cultivation and the PCR analysis. The bacterial cultures revealed the growth of *Mycobacterium spp.* colonies in 58 (70%) of all of the wild animals, but the PCR testing demonstrated paratuberculosis only in one (7.69%) of the roe deer population.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*, wildlife health, Slovakia

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Introduction

Paratuberculosis (Johne's disease) is a chronic infectious disease characterized by granulomatous enteritis, unstoppable diarrhoea and wasting syndrome that predominantly occurs in ruminants, but which has also been noted in various other species (Münster et al. 2013, More et al. 2017, Szteyn et al. 2017, Fox et al. 2018, Whittington et al. 2019). Paratuberculosis host range in ruminants is broad and actually it is believed that all ruminant species are susceptible. Infection is most common in animals less than 6 months of age, but clinical signs rarely occur in animals less than 2 years of age (Whittington and Windsor 2009). The causative agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Rhodes et al. 2014, Waddell et al. 2016, Rathnaiah et al. 2017). In the host organism, MAP is able to reside and survive inside macrophages by shifting the immune response from Th_1 to Th_2 and suppression of IL-12p40 and iNOS gene expression (Sommer et al. 2009). Since being inside of the macrophages, the MAP is not only protected from humoral immune response of the host, but also the shedding of bacteria in the faeces is minimal; thus the possibility for the detection of the disease is limited (Ganusov et al. 2015). Nevertheless, this stage of the disease is without clinical signs and only tests based on the cell-mediated immune response might be able to detect the disease (Stewart et al. 2004, Li et al. 2017). Massive shedding of MAP occurs in the third and fourth stage of the disease (terminal phase), when profuse diarrhoea and emaciation are present (Koets et al. 2015). Besides that, certain animals remain asymptomatic and continue to shed the organism. In association with the high resistance of MAP in the environment (Richardson et al. 2019) and high resistance to standard water disinfection methods (Naser et al. 2014), management of this epidemic is complicated (Samba-Louaka et al. 2018) and the risk of disease spreading increase (Elliott et al. 2015). Therefore, in combination with the potential high economic impact, paratuberculosis is defined as an important infectious disease of ruminants.

In Europe, beside in ruminants, paratuberculosis has been detected in various wild species like wild boar (*Sus scrofa*), wild rabbit (*Oryctolagus cuniculus*), european brown hare (*Lepus europaeus*), red fox (*Vulpes vulpes*), eurasian badger (*Meles meles*), stoat (*Mustela erminea*) weasel (*Mustela nivalis*) and numerous rodent species (Beard et al. 2001, Machackova et al. 2004, Shaughnessy et al. 2013, Toth et al. 2013). Since in Slovakia, there are no official data on the prevalence of MAP in wildlife, except brown bears, waterfowl and terrestrial birds, the purpose of our study was to determine the presence of MAP in various wildlife species originating from the areas where paratuberculosis was detected in domestic ruminants.

Materials and Methods

Sampling

During a four-year period, samples of spleen, liver, kidney, lungs, caecum, small and large intestine, ileocecal valve and mesenteric lymphatic nodes from 83 animals representing 13 species, including european bison (*Bison bonasus*), mouflon (*Ovis musimon*), chamois (*Rupicapra rupicapra tatrica*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), wild boar (*Sus scrofa*), grey wolf (*Canis lupus*), red fox (*Vulpes vulpes*), wildcat (*Felis silvestris*), stone marten (*Martes foina*), eurasian badger (*Meles meles*), alpine marmot (*Marmota marmota*); representing three orders i.e. Artiodactyla, Carnivora and Rodentia were collected. Clasification of each species to family and order is shown in Table 1. The samples were taken and the laboratory analyses were accomplished between 2016 and 2019. All animals were shot during execution of regular game management plans and samples were presented by the kindness of the hunters or were found dead. No animals showed clinical signs of paratuberculosis. In addition, no necropsy revealed any gross lesions characteristic for paratuberculosis. Each sample was stored, properly signed, cooled down and transported to the lab where it was frozen to -70°C until the analysis.

Cultivation of MAP

Each sample was excised (10g) and triturated in the mortar containing 2 ml PBS. 100 µl of the suspension was inoculated in the tubes containing Löwenstein-Jensen medium supplemented with mycobactin (Oxoid). The tubes were incubated for nine weeks with regular screening for mycobacterial growth on the third, sixth and ninth week.

DNA isolation

Suspected colonies were picked up and resuspended in pure MILIQ water. Tubes with colonies were boiled for 5 min and the supernatant was gained after centrifugation. The total DNA was isolated from the supernatant by using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's procedures.

PCR analysis

Nested PCR with two specific sets of primers were used for increased sensitivity and specificity of the detec-

Table 1. Classification of species.

Order	Family	Species
Artiodactyla	Bovidae	european bison (*Bison bonasus*)
		mouflon (*Ovis musimon*)
		chamois (*Rupicapra rupicapra tatrica*)
	Cervidae	roe deer (*Capreolus capreolus*)
		red deer (*Cervus elaphus*)
		fallow deer (*Dama dama*)
	Suidae	wild boar (*Sus scrofa*)
Carnivora	Canidae	grey wolf (*Canis lupus*)
		red fox (*Vulpes vulpes*)
	Felidae	wildcat (*Felis silvestris*)
	Mustaelidae	stone marten (*Martes foina*)
		eurasian badger (*Meles meles*)
Rodentia	Sciuridae	alpine marmot (*Marmota marmota*)

Table 2. Results of cultivation and nested PCR detection of MAP in Slovakian wildlife animals according species.

Species	Number of analysed animals	Presumptive positive colonies	PCR positive
European bison (*Bison bonasus*)	1	1	0
Mouflon (*Ovis musimon*)	2	2	0
Chamois (*Rupicapra rupicapra tatrica*)	12	7	0
Roe deer (*Capreolus capreolus*)	13	10	1
Red deer (*Cervus elaphus*)	10	7	0
Fallow deer (*Dama dama*)	6	5	0
Wild boar (*Sus scrofa*)	13	7	0
Grey wolf (*Canis lupus*)	2	1	0
Red fox (*Vulpes vulpes*)	13	11	0
Wildcat (*Felis silvestris*)	2	2	0
Stone marten (*Martes foina*)	1	1	0
Eurasian badger (*Meles meles*)	5	4	0
Alpine marmot (*Marmota marmota*)	3	0	0
TOTAL	83	58	1

tion method. The primers were designed as previously described by Bhide et al. (2006). In the first round, MPF1 (5′-AGGGTGTTCGGGGCCGTCGCTTAG-3′) and MPR1 (5′-TGAGGTCGATCGCCCACGTGACCT-3′) primers which produced an amplicon of 406 bp. In the second round, MPF2 (5′-ATGTGGTTGCTGT GTTGGATGG-3′) and MPR2 (5′-CCGCCGCAATCA ACTCCAG-3′) primers were used for amplification of 298 bp fragment. Modified protocol was performed according with Bhide et al. (2006). Briefly, first round conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 75 s, annealing at 56.5°C for 50 s and extension at 72°C for 60 s with a final extension at 72°C for 10 min; second round conditions were similar, only the annealing temperature was 63°C for 45 s. After the PCR amplification, all samples were analysed by electrophoresis in 1.2% agarose gel after staining with ethidium bromide.

Statistical analysis

The number of samples was small and no sex determination was done. Therefore, we counted the individual species according to orders and families and statistically compared them. In the further analysis only two orders with a larger number of species and examined individuals, i.e. Artiodactyla and Carnivora, were statistically tested.

All statistical analyses were performed using MS Excel 2003 for Windows XP and the statistical analysis

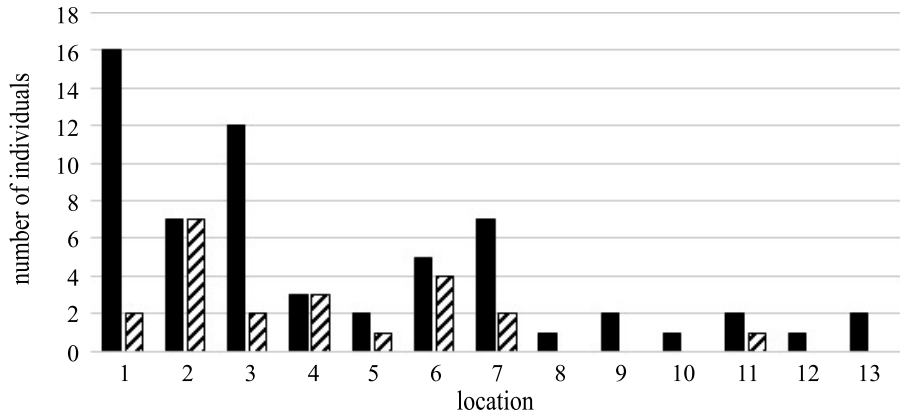

Fig 1. Number of positive (black columns) and negative (striped columns) samples for mycobacterial growth, according to the location. 1 - Rozhanovce, 2 - Tatranský Národný Park, 3 - Hanušovce nad Topľou, 4 - PZ Družba, 5 - Remeniny, 6 - Makovica, 7 - Ruskov, 8 - Revúca, 9 - Slanská Huta, 10 - Trebišov, 11 - Ždaňa, 12 – Národný Park Poloniny, 13 - Nižna Myšla.

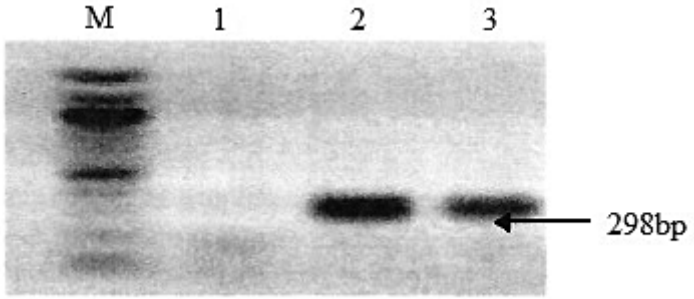

Fig 2. Positive result of electrophoresis. Visualization of 298bp length fragment of Mycobacterium avium subsp. paratuberculosis after nested PCR in one roe deer sample.
Legend: Lane M: Standard of molecular weights (100 bp standard), Lane 1: negative control for MAP, Lane 2: MAP positive sample, Lane 3: positive control for MAP

system GraphPad Prism, version 5.01 (GraphPad Software, Inc., San Diego, California, USA). Both, Fisher's exact test and the chi-square test (χ^2) were used. Fisher's exact test was used in comparisons between two orders i.e. Artiodactyla and Carnivora. Subsequently, we also compared families for both orders. Values of p less than 0.05 were considered as significant.

Results

The results from 83 individuals of 13 wildlife species investigated for the presence of *Mycobacterium avium* subspecies *paratuberculosis* are shown in Table 2. Following cultivation, samples from 58 (70%) animals were suspected for growth of mycobacterial colonies. The percentage of animals suspected for mycobacterial growth (sample size >10) was red fox (85%), roe deer (77%), red deer (70%), chamois (58%) and wild boar (54%). Positive/negative samples according to the location are presented in the Fig. 1. Only one sample, ileocecal valve of a female roe deer (7.69%), was PCR-positive (298bp fragment) for MAP (Fig. 2).

The comparison of MAP prevalence between orders regardless to species confirmed non-significant difference (p=0.272 by Fisher's exact test). Chi-square test (χ^2) was used for the comparison of MAP prevalence between families. For the Artiodactyls, non-significant difference was confirmed among them, i.e. higher than 0.05 (χ^2=2.04, df=2, p=0.360). For the Carnivore, non-significant difference was shown among them (χ^2=0.494, df=2, p=0.781).

Discussion

Paratuberculosis in wildlife is generally a problem in captive animals, mainly farmed red deer (Godfroid et al. 2000, de Lisle et al. 2003, Kopecna et al. 2008a) and, as was recently confirmed, in animals in zoological gardens (Münster et al. 2013). Previous studies reported antibody seroprevalence in roe deer ranging from 22% (n=40) in North-western Italy, 12.2% (n=49) in Norway, 9.2% (n=519) in Spain, to only 0.9% (n=245) in southern France and 0.2% (n=835) in the Czech Republic (Tryland et al. 2004, Kopecna et al. 2008b, Robino et al. 2008, Boadella et al. 2010, Candela et al. 2014). Pavlik et al. (2000) made a large-scale epidemiological survey of MAP in wild ruminants in the Czech Republic and detected it (culture and PCR) in the 3.5%

of samples collected from free-living animals, including 1.05% of red deer, 2.6% of fallow deer, 2.2% of roe deer and 2.9% of mouflons. Sarno et al. (2013) analysed 198 faecal samples of red deer, roe deer, chamois and ibex, none of them were PCR-positive for MAP. In general, the mean MAP prevalence in wildlife was reported at 2.41% (95% CI 1.76-3.06) which is rather low (Carta et al. 2012, Carta et al. 2013). On the other hand, Krzysiak et al. (2014) noticed no seropositive reaction for MAP in 23 investigated European bison in Northern-East Poland. Paratuberculosis in Slovakian wildlife has been detected only in 2/20 (10%) of the brown bears (*Ursus arctos*) in the Low Tatras Mountains up to now (Kopecna et al. 2006). Furthermore, Gronesova et al. (2008) confirmed 8/109 (7.34%) positive samples for MAP in waterfowl and terrestrial birds (Common Starling - *Sturnus vulgaris*; Black-headed Gull - *Larus ridibundus*; European Curlew - *Numenius arquata*; Ruff - *Philomachus pugnax*; Common Cuckoo - *Cuculus canorus*; Savi's Warbler - *Locustella luscinioides*). On the contrary Borovská et al. (2011) affirmed all 650 investigated bird's samples were negative for MAP. Our study is the first report which confirmed MAP in 7.69% in roe deer in Slovakia, but the sample size was rather small (n=13).

The impact of MAP on wild populations remains dubious, especially when it is known that different strains in different hosts can influence the development of the disease as well as its future clinical presentation (Abendaño et al. 2013). However, Barkema et al. (2018) clearly ranked the lack of knowledge about the prevalence of MAP in wildlife among the most important factors that hamper prevention and control of MAP infections. In addition, they described other factors including the knowledge of MAP prevalence in wildlife which can contribute to clarify the impact of infections on the wildlife health and moreover to clarify the role of wildlife as a MAP reservoir and in transmitting MAP to livestock. Understanding the potential impact of paratuberculosis in wildlife requires the detection of the bacterial serotypes. In red deer, the resistance to clinical diseases seems to be age-related, with the younger ones being more susceptible (Mackintosh et al. 2010).

According to other studies, the prevalence of MAP-positive animals seems to be related to the numbers of cattle and use of liquid manure on pastures (Daniels et al. 2003, Raizman et al. 2005, Nugent et al. 2011). As roe deer are non-gregarious browsers with a small home range, it seems that the prevalence of MAP in this species is largely influenced by factors in the environment. The ecological modelling results of Cunha et al. (2020) suggested that the probability of MAP infection may be connected with lower annual rainfall, as well as with higher altitude and temperature stability. However, the density of livestock farms was not a significant predictor.

Paratuberculosis causes direct and indirect economic losses, impacts animal welfare and also arouses public health concerns. Our findings confirmed the presence of *Mycobacterium avium* subspecies *paratuberculosis* in wildlife in Slovakia and suggested that they may be an important reservoir for transmission of MAP in domestic ruminants. Further studies with larger sample sizes seems to be necessary, also for improving effectivity of the control program of MAP in cattle.

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