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Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples of hunted deer, chamois and ibex in Switzerland

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Vorkommen von *Mycobacterium avium* subsp. *paratuberculosis* in Kotproben von gejagten Rehen, Hirschen, Steinböcken und Gämsen in der Schweiz

23

24 **Introduction**

25 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiological agent of bovine
26 paratuberculosis, a chronic granulomatous enteritis, also known as Johne's disease. MAP
27 prevalence in domestic food producing animals is increasing globally, causing significant
28 economic losses to the livestock industries through subclinical effects (reduced milk yield and
29 weight loss) and subsequent death of the affected animals. Moreover, several reports suggest
30 a possible link between human Crohn's disease and MAP due to remarkable clinical,
31 epidemiological and pathological similarities (Chacon *et al*., 2004), but a causal relationship
32 has not yet been proven. Nevertheless, the possible involvement in human disease has led to
33 an increased awareness of these microorganisms as public health concerns.
34 The natural hosts for MAP are domestic ruminants with higher prevalence in dairy and beef
35 cattle herds around the world (Manning and Collins, 2001). For Switzerland, different studies
36 showed a low prevalence of MAP in the cattle population (Glanemann *et al*., 2004; Bosshard
37 *et al.,* 2006).
38 Wild ruminants may act as a reservoir and contribute to the spread of MAP through fecal
39 shedding (Manning, 2011). Their interaction with susceptible farmed ruminants raises the
40 possibility of playing a role in the epidemiology of the disease in domestic livestock. An
41 interspecies transmission between cattle and wild-living red deer (*Cervus elaphus*) has been
42 recently proposed by multitarget genotyping of strains from both animal species (Fritsch *et al.,*
43 2012). However, reported detection rates in wild ruminants vary among different surveys.
44 From fecal samples of red deer a prevalence of 0.1% has been reported in a Czech Republic
45 survey (Pribylova *et al*., 2011), whereas a much higher prevalence was found in India where
46 15 % of hog deer (*Axis porcinus*) and 57 % of gaur fecal samples (*Bos gaurus*) were tested
47 positive for MAP (Singh *et al.,* 2010).

48 In view of detection techniques, several culture methods without decontamination or
49 combining chemical or antibiotic decontamination steps and selective media have been
50 proposed (Glanemann *et al*., 2004; Akineden *et al*., 2011). However, these methods are time
51 consuming. Immunological-based systems are faster than culture methods but have a low
52 sensitivity (Glanemann *et al*., 2004; Collins *et al.,* 2005). Molecular based-methods such as
53 PCR assays may represent a rapid screening system for MAP detection. Most of these
54 protocols are based on the IS*900* insertion elements but IS*900*-like sequences found in other
55 Mycobacteria can lead to false positive results. Therefore other sequences without
56 homologues in other Mycobacteria have been proposed; ISMap*02* is present in six copies
57 (Stabel and Bannantine, 2005), IS*Mav2* is present in three copies (Li *et al*., 2005), and F57
58 and Hsp X both occur as single copy genes (Tasara and Stephan, 2005). Nevertheless, these
59 methods have a lower sensitivity compared to the cultural based methods and may not detect
60 subclinically infected animals (low shedders). The objectives of this study were a) to
61 investigate the occurrence of MAP in fecal samples collected from hunted wild ruminants by
62 a combination of cultural detection and PCR based identification and b) to asses the impact of
63 wild ruminants in the spread of MAP in Switzerland.
64
65 **Sampling**
66 During September to November 2011, state gamekeepers and hunters collected immediately
67 after shooting and evisceration, *in loco*, fecal samples from wild ruminants. For each animal,
68 sex, age, and location of hunting were recorded. Fecal matter (approximately 10 grams per
69 sample) was collected from the colon after the large intestine had been opened, placed into
70 sterile tubes and sent to the laboratory where samples were stored at -20°C until processing.
71
72 **Detection of MAP**

73 For MAP detection, fecal samples from 3 animals of the same species were pooled (total
74 weight of 6g). Each pooled sample was first homogenized in 8ml of sterile demineralized
75 water and stored under chilled conditions for 4 h. Thereafter, samples were decontaminated
76 using a NAOH/oxalic acid treatment (Beerwerth,1967). Briefly, samples were mixed in 50 ml
77 of a 4% NaOH solution and subsequently transferred into a sterile centrifugation tube and
78 shaken for 8 min on an orbital shaker (Bühler, Hechingen, Germany). After centrifugation
79 (5010 rpm, 15 min, 20°C) the pellet was resuspended in 20 ml of a 5% oxalic acid solution
80 and shaken for 15 min on an orbital shaker. Tubes were centrifuged a second time as
81 described before; the pellet was resuspended in 6ml of sterile 0.9% saline and used as
82 inoculum. For each sample Herrold's Egg Yolk Agar with Mycobactin J and ANV (HEYA)
83 (Becton, Dickinson and Company, Sparks, MD, USA) in duplicates were inoculated with an
84 aliquot (200 µl) of the final processed and decontaminated material. Slants were incubated at
85 37°C and monitored visually for growth of presumptive positive MAP colonies (small whitish
86 colonies slightly elevated from the surface) every two weeks for up to 16 weeks. Presumptive
87 positive colonies were recorded, subcultured in Middlebrook 7H9 broth supplemented with
88 Middlebrook-ADC growth supplement (Becton, Dickinson and Company, USA) and 2 mg/L
89 Mycobactin J (Synbiotics Europe SAS, Munich, Germany), and incubated at 37°C for 1 week.
90 After Ziehl-Neelsen staining, subcultures with red acid-fast rods were finally confirmed as
91 MAP by using an F57 sequence-based Real-Time PCR protocol (Bosshard *et al.,* 2006).
92
93 **Results and Discussion**
94 In total 198 fecal samples (69 red deer (*Cervus elaphus*), 51 roe deer (*Capreolus capreolus*),
95 51 chamois (*Rupicapra rupicapra*), and 9 ibex (*Capra ibex*)) were collected. The majority of
96 the hunted animals were on average 2 years old and originated from the central and eastern
97 part of Switzerland. Using the described methods, none of the pooled fecal samples from the
98 wild ruminants were found to be positive for viable MAP (Tabl 1).

99 Detection was based on phenotypic properties (small whitish colony morphology and slow
100 growth) and Mycobactin J dependency. Culture-positive HEYA slants (MAP-like colonies)
101 were found in 19 out of 66 decontaminated fecal pools (11 red deer, 7 roe deer and 1
102 chamois). None of the ibex samples showed bacterial growth. Red acid fast rods forming
103 cluster from culture-positive HEYA slants were visually detected by Ziehl-Neelsen staining
104 from 10 red deer, 4 roe deer and 1 chamois, respectively, but finally confirmed as non-MAP
105 cells by F57 PCR.
106 Even though competitive growing microorangisms and chemical treatments might hinder and
107 reduce growth of small numbers of viable MAP cells (Grant *et al*., 2003), the cultural
108 technique is still considered the "reference standard" for detection of MAP in fecal samples.
109 Based on the results of this first study, a low occurrence of MAP in wild ruminants in
110 Switzerland can currently be postulated. Therefore, wild ruminants do not seem to have a big
111 impact in spreading MAP to farmed ruminants. Nevertheless, further studies covering all
112 regions of Switzerland and including a higher number of animals are necessary to get a
113 complete picture of the impact of wild ruminants in the epidemiology of MAP.
114
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117 animals.
118
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191 **Table 1:** Origin and MAP detection results of the 198 fecal samples collected from wild

192 ruminants during the hunting season 2011

193

Species	No. Samples	No. Pools	No. of pools with growth of presumptive positive colonies	No. of F57 PCR confirmed MAP positive samples
Red deer	69	23	11	0
Roe deer	51	17	7	0
Chamois	51	17	1	0
Ibex	9	9	0	0
Total	198	66	19	0

194