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Anaplasma phagocytophilum and *Babesia* spp. in roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*) and mouflon (*Ovis musimon*) in Germany

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ABSTRACT

Infections with the tick-borne pathogens *Anaplasma phagocytophilum* and *Babesia* spp. can cause febrile disease in several mammalian species, including humans. Wild ruminants in Europe are suggested to serve as reservoir hosts for particular strains or species of these pathogens. The aims of this study were to investigate the occurrence of *A. phagocytophilum* and *Babesia* spp. in roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*) and mouflon (*Ovis musimon orientalis*) in Germany, and the diversity and host association of genetic variants of *A. phagocytophilum* and *Babesia* species. From 2009 to 2010, 364 spleen samples from 153 roe deer, 43 fallow deer and 168 mouflon from 13 locations in Germany were tested for DNA of *A. phagocytophilum* and *Babesia* spp. by real-time PCR or conventional PCR, respectively. Variants of *A. phagocytophilum* were investigated with a nested PCR targeting the partial *16S rRNA* gene, and species of piroplasms were identified by sequencing. DNA of *A. phagocytophilum* was detected in 303 (83.2%) samples: roe deer, 96.1% (147/153); fallow deer, 72.1% (31/43); and mouflon, 74.4% (125/168). Sequence analysis of *16S rRNA*-PCR products revealed the presence of nine different genetic variants. DNA of *Babesia* spp. was found in 113 (31.0%) samples: roe deer, 62.8% (96/153); fallow deer, 16.3% (6/43); and mouflon, 6.5% (11/168). *Babesia capreoli, Babesia* sp. EU1 (referred to also as *B. venatorum*), *B. odocoilei*-like and a *Theileria* species were identified. Co-infections with *A. phagocytophilum* and *Babesia* spp. were detected in 30.0% of the animals which were tested positive for *A. phagocytophilum* and/or *Babesia* spp. Roe deer had a significantly higher percentage of co-infections (60.8%), followed by fallow deer (14.0%) and mouflon (6.5%). Thus, the results suggest that roe deer plays a key role in the endemic cycles of the pathogens investigated.

1. Introduction

The distribution of tick-borne pathogens depends on the occurrence of ticks, tick hosts and competent reservoirs. Wild ungulates constitute important hosts for ticks and, thus, play a critical role in the life cycles of ticks as well as in the transmission cycles of several tick-borne pathogens [1]. The main vector for the obligate intracellular bacterium *Anaplasma phagocytophilum* and several *Babesia* species in Europe is the most common hard tick *Ixodes ricinus*. These pathogens are of increasing importance in Europe [2].

Whilst *A. phagocytophilum* is one of the most common tick-borne disease agents of humans in USA, it is rarely detected in humans in Europe, despite that it being commonly detected in ticks in this geographic region [3]. Human babesiosis is considered as an emerging zoonosis in Europe [4] and, in the last decade, two clinical cases of human babesiosis caused by *B. microti* and *Babesia* sp. EU1 (referred to also as *B. venatorum*), respectively, have been reported

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[5,6]. In Europe, evidence of *A. phagocytophilum* infection or previous contact has been established for a large variety of wild mammalian species [7,8] Among these animals are several species of rodents, insectivores and hares (*Lepus europaeus*) [9–13], wild carnivores, such as the red fox (*Vulpes vulpes*), Eurasian lynx (*Lynx lynx*), timber wolf (*Canis lupus lycaon*) and racoon dog (*Nyctereutes procyonoides*) [14–18], brown bear (*Ursus arctos*) [19], and several wild ungulates, such as wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), moose (*Alces alces*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), European bison (*Bison bonasus*), feral goat (*Capra aegagrus hircus*), Alpine ibex (*Capra ibex*) and chamois (*Rupicapra rupicapra*) [20–27]. *Anaplasma phagocytophilum* is a genetically heterogeneous species as has been shown on the basis of different genes, such as *16S rRNA*, *groEL*, *msp4*, *msp2* and *ankA*, and several housekeeping genes [28–30]. It was suggested that variation may be associated with host preference or pathogenicity, or might depend on the geographical distribution [8,24,29–31].

Currently several *Babesia* species have been detected in wild ungulates in Europe: *B. capreoli, B. divergens, B. bigemina, B. motasi, B. ovis* and *Babesia* sp. EU1 [27,32–38]. In addition, evidence was provided of genetic variants, such as a *B. odocoilei*-like taxon in red deer or *Babesia* sp. MO1 and *Babesia* sp. CH1 in roe deer, respectively [35,38].

Systematic studies of the occurrence of *A. phagocytophilum* and *Babesia* species in wild ruminant populations covering a large geographic area from Germany are lacking. However, based on previous studies from Alpine regions in Austria and Switzerland as well as from Southern Germany [26,27,37,38], it has been hypothesised that these tick-borne pathogens occur also more widespread in wild ruminant populations in Germany.

The aims of this study were to investigate the presence of *A. phagocytophilum* and *Babesia* spp. in roe deer, fallow deer and mouflon in Germany with particular attention to the occurrence of the pathogens in sympatric populations of roe deer and mouflon. In addition, the occurrence of *A. phagocytophilum* variants based on the variation in the partial *16S rRNA*-gene as well as the diversity of *Babesia* species and potential host-pathogen associations were investigated.

2. Materials and methods

2.1. Sampling

Spleen samples from roe deer, fallow deer and mouflon (*Ovis musimon orientalis*) were donated by hunters from 13 locations in seven federal states in Germany (Fig. 1) during the hunting seasons from May 2009 to November 2010. Eleven of the locations had sympatric roe deer and mouflon populations, and one each had fallow deer or mouflon only. Spleen samples (1 cm^3) were taken from individual animals using a sterile tissue punch (for each sample) and preserved in 70% ethanol in 15 ml Falcon tubes. To prevent contamination, collectors were provided with individually labelled materials and with detailed instruction on the sampling procedure. Spleen samples were collected from a total of 364 wild ungulates (153 roe deer, 168 mouflon and 43 fallow deer). Information on the sex and age of the animals was also collected (Table 1); animals were stratified into three age groups: <1 year, 1–2 years and >2 years.

2.2. DNA extraction

The extraction of genomic DNA from the spleen was carried out with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction for animal tissues. Samples were incubated over night at 56 °C and eluted twice with 100 µl of buffer each time. DNA content and quality were assessed using a spectrophotometer (NANODROP® ND-1000, Peqlab. Erlangen, Germany). Samples with high amounts of DNA (exceeding 100 ng/µL) were diluted (1:2) using elution buffer.

2.3. Polymerase chain reaction (PCR)

All samples were screened for the presence of DNA of *A. phagocytophilum* using a real-time PCR, targeting a 77 bp region of the *msp2* gene, as described previously [27]. Two to eight *A. phagocytophilum* test-positive samples from each location (depending on the number of samples available from a particular location) were selected and a PCR targeting of a 497 bp part of the *16 S rRNA* gene was performed as described elsewhere [28,39]. For the detection of *Babesia*-DNA, a PCR targeting a part of the *18S rRNA* gene was performed as described previously [40,41]. The sequence of this part of the gene allows the discrimination between *B. capreoli* and *B. divergens* [42]. To further characterize the *Theileria* spp., which had been amplified in the *Babesia*-PCR, a conventional PCR targeting a 650 bp part of the *18S rRNA* gene of *Theileria* spp. was conducted [43]. Primers and probe are shown in Table 2.

2.4. Agarose gel electrophoresis

Products derived from conventional PCRs were examined by gel electrophoresis (2% agarose gel, Top Vision Agarose, Fermentas, Germany) and stained with 0.01% GELRED™ (Biotium, Hayward, California) and detected using ultraviolet light (PeqLab, Erlangen, Germany).

2.5. Sequence analysis

PCR products were purified using minicolumns (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol and sent to an external laboratory (Eurofins MWG Operon, Ebersberg, Germany) for sequencing in both directions using the same primers as employed in PCR. The quality of the sequences was analysed with CHROMASLITE® and reverse sequences were reverse complemented (http://www.bioinformatics.org/sms/rev_comp.html). Forward and reverse sequences were assembled with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Sequences were compared with sequences available from the GenBank® database with the BLASTn tool of the National Center for Biotechnology (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Statistical analysis

Data were entered in MSExcel tables (Microsoft Corporation, Redmont, Seattle, USA) and statistical analyses were performed using the program Prism 5 (GraphPad Prism®, La Jola, California, USA). Contingency table analysis using χ^2-Test and Fisher's exact test was performed to test for associations of the prevalence of pathogens with host species, host age and gender or between pathogens. A significance level of $p = 0.05$ was used. Samples that tested positive for *Theileria* were not considered in the statistical analysis.

3. Results

3.1. Anaplasma phagocytophilum

Spleen samples from 303 of 364 (83.2%) wild ungulates tested positive for *A. phagocytophilum* DNA. *Anaplasma phagocytophilum* DNA was detected in all host species: 147 of 153 roe deer (96.1%), 31 of 43 fallow deer (72.1%) and 125 of 168 mouflon (74.4%) (Table 3).