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Contribution of tick-borne diseases to mortality in juvenile free-living cervids

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Abstract

Introduction and objective. Reports on tick-borne infections in free-living juvenile animals and their impact on survival of cervids in nature are lacking. The aim of the study was to detect and identify the *Babesia* and *Anaplasma phagocytophilum* species/ecotypes that may have contributed to the death of juvenile animals from a wildlife rescue centre in spring 2020.

Materials and method. PCR amplification and sequencing of two genetic markers (18S rDNA and *cox1* for *Babesia*, 16S rDNA and groEL for *A. phagocytophilum*) were used for screening eleven samples derived from juvenile animals which died in a rescue centre (seven roe deer *Capreolus capreolus*, one elk *Alces alces*, one red squirrel *Sciurus vulgaris*, one European beaver *Castor fiber*, one red fox *Vulpes vulpes*). Phylogenetic analysis of full-length 18S rDNA sequence was performed to enable differentiation between two closely-related species infecting wild ungulates, *Babesia capreoli* and *Babesia divergens* (zoonotic).

Results. The occurrence of the typical SNPs of *B. capreoli* at two discriminating positions in the 18S rRNA gene allowed identification of *B. capreoli* infection in a roe deer calf. In two calves, *Anaplasma phagocytophilum* ecotype 2 was identified, including the same calf co-infection. No *Babesia* DNA was amplified in an elk calf treated for babesiosis. Splenomegaly was recorded in roe deer calves with *A. phagocytophilum* and *A. phagocytophilum* + *B. capreoli* infection.

Conclusions. The study revealed that tick-borne infections are common among juvenile, free-living cervids and may contribute to their mortality.

Key words

Anaplasma phagocytophilum, roe deer, *Babesia capreoli*, Eurasian elk, fatal babesiosis

INTRODUCTION

Ungulates, especially cervids (*Cervidae*), are key players in some host-vector–pathogen interactions, acting as reservoir hosts for many pathogens and as the main source of blood meals for *Ixodes ricinus* females [1, 2].

In Poland, roe deer (*Capreolus capreolus*) is the most abundant member of the *Cervidae* family [3, 4, 5]. Both roe deer and Eurasian elk (*Alces alces alces* Linnaeus, 1758) are classified as a game species; however, since 2001, elk have been protected by law throughout the whole year (Dz. U. 2001, No. 43, position 488). This species was almost exterminated after World War II after which it was actively protected and its survival aided by being reintroduced in some parts of the country, i.e., in Central Poland, in the Kampinoski National Park near Warsaw in 1951 (https://www.kampinoski-pn.gov.pl/przyroda/fauna). Since 2001, elk populations have grown and spread considerably in Poland, reaching a population size of some 23,700 individuals by 2019 [5]. Eurasian elk is still considered a rare and protected species, and our knowledge of the natural diseases that limit their survival in nature is still insufficient [6, 7].

Generally, the impact of vector-borne diseases, including tick-borne babesiosis, on populations of free-living mammals is still poorly understood due to the difficulties inherent in monitoring naturally infected wild individuals. In some populations, i.e. in the red fox, tick-borne infections are very abundant and their impact on survival appears to be generally modest [8, 9]. However, to our knowledge, the contribution of tick-borne diseases to mortality among juvenile wildlife in general, and cervid calves particular, has not been intensively investigated. The current study was performed following information on the death of an elk calf which died in a rescue centre, having presented with clinical signs of acute babesiosis. Molecular techniques were used to detect the presence of tick-borne pathogens among juvenile mammals which died despite treatment in this centre in spring 2020.

MATERIALS AND METHOD

Ethical issue. No animals were culled for this study. Since the study was carried out on blood and tissue samples provided by the veterinary staff of a wildlife rescue centre, no ethical approval/ license was required (as per the Resolution on the Protection of Animals used for Scientific or Educational Purposes, 15 January 2015: Dz. U. 2015 position 266, Chapter 1, Paragraph 1.2.1).

In July 2020, eleven blood and spleen samples were obtained from a wildlife rescue centre in Central Poland. The samples were derived from seven juvenile roe deer and one elk calf (Tab. 1). The deer calves died likely due to serious injuries and trauma (n=7), mostly attributable to traffic collisions,

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and from unknown reasons (n=2). The elk calf died of suspected babesiosis. Additionally, one juvenile European beaver (*Castor fiber*), one red squirrel (*Sciurus vulgaris*) and one red fox pup were screened.

Elk – case description. A female elk calf about two weeks old was found abandoned, weakened and tick-infested. The calf was observed over a three-day period, with no signs of its mother it had sought the company of horses. At clinical presentation on the 7 June 2020, the calf was emaciated and dehydrated. It was immediately administered intravenous fluids (0.9% saline, electrolytes [Optylite]), and was fed with goat milk every two hours. A few days later, the calf developed minor diarrhoea and was treated with diosmectite, with additional herbal extract (chamomile), oral administration of linseeds and fluid therapy. Antibiotic treatment consisting of amoxicillin (Clamoxyl 150 mg/ml) was administered to counter secondary infections. On the 16 June, an apparent improvement in its physical condition was noticed: the calf resumed normal appetite (diet consisting of goat milk, hay, crushed oats and tree twigs *ad libidum*), and was very active.

On the afternoon of 22 June, the calf presented with apathy, anorexia and an ataxic gait. Body temperature was 40.6 °C. *Babesia* infection was suspected and treatment with imidocarb dipropionate (Carbesia, MSD Intervet; in a single dose recommended for bovines: 0.85 mg/1 kg) was initiated. The calf received also intravenous fluids, amoxicillin (intravenous Clamoxyl 150 mg/ml) and methylprednisolone (Solu-Medrol 250 mg/4 ml).

Temporal improvement was observed during the following night, with a reduction in body temperature (37.9 °C), and an increase in activity and appetite. On the morning of 23 June, the calf received fluid supply and Catosal 10%; however, by the early afternoon of the same day the animal suffered a rapid relapse and babesiosis symptoms were observed: calf was recumbent, refused food, its body temperature was 39.0 °C, and the mucous membranes were pale. Despite continuous intravenous fluid therapy, the calf died a few hours later.

Laboratory diagnostics. DNA was extracted from 100 µl of blood and 25mg of spleen samples by use of Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), respectively, following the manufacturer's instructions.

For the detection of *Babesia* spp., a single-step PCR was used to amplify the 560 bp 18S rRNA gene fragment of *Babesia/Theileria* [10, 11]. For genotyping of *Babesia*-positive samples, an almost a full-length 18S rDNA (1700 bp) was amplified with apicomplexan 18S rRNA-specific primers Nbab1F 5′-AGCCATGCATGTCTAAGTATAAGCTTTT-3′ [12] and TB Rev 5′-AATAATTCACCGGATCACTCG-3′ [13] encompassing two of three base positions (positions 631 and 663 of the full gene), described previously as discriminating between *B. capreoli* and *B. divergens* [14]. Positive samples were sequenced by a private company (Genomed S.A., Warsaw, Poland).

For the phylogenetic analysis of *Babesia*, the Akaike information criterion was used in the jModel Test to identify the most appropriate model of nucleotide substitution. A representative tree for 18S rDNA of *Babesia* spp. was constructed using MEGA v. 7.0, by the Maximum Likelihood method and Kimura 2-parameter model. Additionally, to detect *Babesia* infection in elk, samples (blood and spleen), a 328 bp fragment of *cox1* gene was amplified, as described previously [9].

The samples were also screened for tick-borne bacteria: amplifying *Borrelia burgdorferi* s.l. (*Borreliella* spp.) *fla* gene sequence, and for *A. phagocytophilum* and Ca. *Neoehrlichia mikurensis* (CNM), amplifying 16S rDNA – *rss* gene fragments, as described previously in detail [15, 16].

Finally, to determine the ecotype of detected *A. phagocytophilum*, *groEL* gene fragment was amplified according to the protocol of Alberti et al. [17]. Using the MAFFT algorithm [18], the A. *phagocytophilum* sequences were aligned with identical sequences from the GenBank database (recognized by BLAST NCBI) and reference sequences from Jahfari et al. [19]. A Maximum Likelihood tree was constructed using automatic parameters on the IQ-TREE web server [20].

RESULTS

DNA of tick-borne pathogens was found only in roe deer samples (Tab. 1). The blood sample from the elk calf tested negative in both PCR protocols for *Babesia* spp. Two roe deer tested positive for *A. phagocytophilum* (2/7=28.6%). One of these calves also tested positive for *Babesia*. Interestingly, splenomegaly was observed during necropsy in these two infected calves (Tab. 1). The calf diagnosed in the present study with co-infection of *Babesia* and *A. phagocytophilum* died from unknown causes, and did not receive any treatment for babesiosis or anaplasmosis.

Table 1. Sample characteristics and tick-borne infections

Sample identifier	Host species and gender	Host weight and age	*Babesia* spp.	*A. phago-cytophilum*	Necropsy findings
R1	Roe deer, female	1.5 kg, few days	-	-	Head trauma
R2	Roe deer, male	2 kg, few days-week	-	-	Left hind leg missing (trauma)
R3	Red squirrel, female	0.5 kg adult	-	-	-
R4	Roe deer, female	1.7 kg, few days	-	-	-
R5	Roe deer, male	4 kg, few weeks	-	-	splenomegaly
R6	European beaver, female	1 kg, juvenile	-	-	Numerous wounds (dog bites)
R7	Roe deer, female	3 kg, few weeks	-	-	Head trauma
R8	Roe deer, female	6 kg, few weeks	-	+	Splenomegaly
R9	Red fox, female	3 kg, several weeks	-	-	Fluid in heart and body cavity
R10	Eurasian elk, female	20 kg, few weeks	+	-	-
R11	Roe deer, male	2 kg, few days	+	+	splenomegaly
Total			2/8 cervids	2/8 cervids	

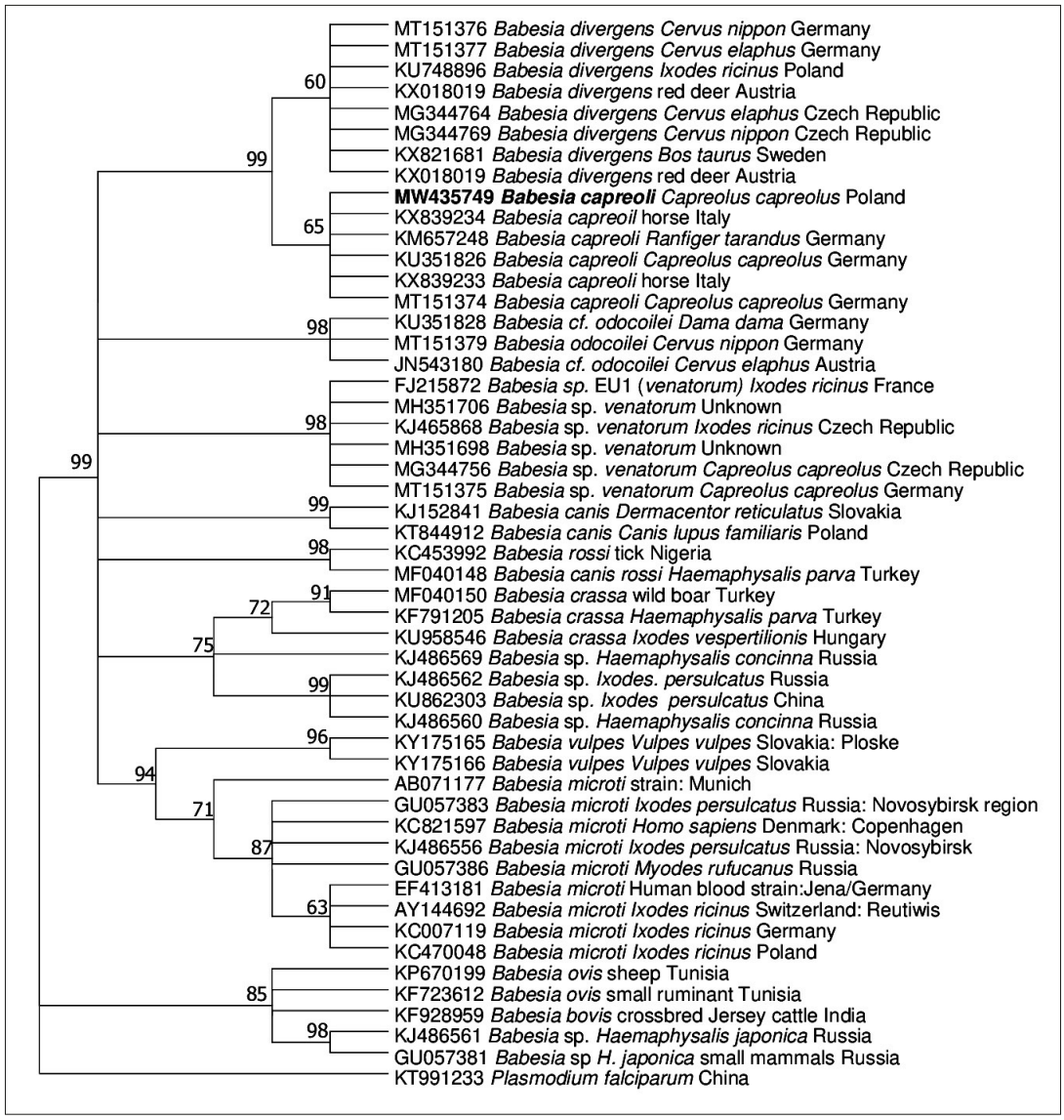

Figure 1. Phylogenetic tree of *Babesia* spp. based on a fragment of the 18S rRNA gene (550bp).
The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-2301.03) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 2.0325)). The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

***Babesia* species identification.** A consensus sequence of 18S rDNA (about 900 bp) displayed 100% similarity to *B. capreoli* (FJ944828) and 99.78% similarity to *B. divergens* (FJ944826). Analysis of discriminating positions (positions 631 and 663 of the full gene) revealed the presence of *B. capreoli* SNPs (G, T). Phylogenetic analysis (Fig. 1) showed that the presented *Babesia* sequence grouped with *B. capreoli* sequences obtained from various deer species (mostly roe deer) from different European countries. The 18S rDNA *B. capreoli* sequence from the roe deer calf was deposited in the GenBank database under Accession No. MW435749.

***Anaplasma phagocytophilum* identification.** Both *rrs* 897 bp consensus sequences showed 100% similarity to two known sequences of *A. phagocytophilum* (HQ629914, AJ242783) and clustered within non-zoonotic lineage 2 of *A. phagocytophilum*, among sequences originating from *I. ricinus* and roe deer in Europe [21]. One *A. phagocytophilum rrs* sequence was deposited in the GenBank under Accession No. MW879362.

One obtained *groEL* sequence (R8) (MZ090590) was identical with *A. phagocytophilum* from *I. ricinus* tick from Estonia (HQ629905) and from roe deer from Switzerland (AF383225), whereas the second sequence (R11) (MZ090591) was identical with *A. phagocytophilum* from *I. ricinus* from Slovenia (KM215250), elk from Sweden (KC800984) and roe deer from Austria (AY220467).

The phylogenetic tree of total 526 sequences (Supplementary Fig. 1) showed that both sequences obtained in the current study belonged to ecotype 2, together with isolates from ruminants, rodents and *Ixodes* ticks of Central and Eastern Europe [19].

All samples were negative for *B. burgdorferi* s.l./*Borreliella* spp. and CNM.

DISCUSSION

The present study has demonstrated that tick-borne infections/co-infections can be identified already in cervid calves only few-weeks old, and could have contributed to mortality despite applied treatment.

During necropsy, an apparent enlargement of the spleen was observed in two roe deer calves, one diagnosed with *A. phagocytophilum* infection alone and one with co-infection of *A. phagocytophilum* and *B. capreoli*. Splenomegaly is one of the common pathological symptoms in *Babesia*- or *A. phagocytophilum*-infected animals and humans; reported in both experimentally- or naturally-infected animals [9, 22, 23]. Among *Babesia* species infecting deer, *B. capreoli* is the most pathogenic and was recently re-described based on comparison with *B. divergens* [14]. Interestingly, this *Babesia* species seems to be abundant not only in roe deer but also occurs sporadically in other deer species (red deer, sika deer, Père David's deer, fallow deer, reindeer) [14, 24, 25, 26, 27]. This piroplasm species can cause acute and fatal disease in roe deer, Père David's deer and reindeer. The clinical signs and necropsy features reported previously in roe deer were typical of babesiosis, and included icterus, haemoglobinuria, haematuria, splenomegaly, haemosiderosis of liver and spleen, and pulmonary oedema [14].

In the current study it was not possible to confirm *Babesia* infection in the elk calf presenting with acute babesiosis. However, this failure could have been attributed to the intensive anti-*Babesia* treatment the calf had received before sampling (see: case description). Veterinary staff at the rescue centre had long-time experience with the diagnosis and treatment of babesiosis as the centre is localized in *Babesia canis* hyper-endemic region resulting with hundreds of babesiosis cases in dogs [28, 29, 30]. Thus, their diagnosis is included here and we may conclude that in addition to road accidents, habitat fragmentation and grey wolf predation [31, 32, 33], tick-borne infections may constitute an additional significant limitation on the growth and spread of elk populations in the region. To date, at least three *Babesia* species (*B. venatorum, B. capreoli*, *B. odocoilei*-like) have been found in Eurasian elk [34, 35].

Anaplasma phagocytophilum detected in two roe deer calves in this study was identified as non-zoonotic ecotype 2, specific to rodents and ruminants, and also found in *Ixodes* ticks [19]. It is plausible that co-infection with *A. phagocytophilum*, known for its immunosuppressive properties and pathogenicity in mammalian hosts [36], could have been a factor triggering fatal systemic breakdown in the identified co-infection with *B. capreoli* in roe deer calf.

Conclusion: Tick-borne pathogens can be found in juvenile cervids and may contribute to mortality among cervid calves.

DECLARATIONS

Ethics approval and consent to participate: not applicable. Consent for publication: not applicable Availability of data and materials: all data generated or analysed during this study are included in this published article. Competing interests: the authors declare that they have no competing interests. Funding: the study was supported by the National Science Centre (NCN) Sonata Bis grant no. 2014/14/E/NZ7/00153 (AB). Authors' contributions: AB conceived the study and prepared ms; AB, MK, MA and DDS performed the sampling and laboratory analyses. DDS and MK performed phylogenetic analyses.

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