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Short Communication

SEVERE *ANAPLASMA PHAGOCYTOPHILUM* AND *BABESIA DIVERGENS* CONCOMITANT INFECTION IN IMPORTED CAPTIVE REINDEER (*RANGIFER TARANDUS*)

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ABSTRACT

Tick-borne diseases are highly prevalent in domestic and wild ruminants and they may be distributed in wide geographical ranges by animal transportation. The aim of the current study was to investigate the presence of European strains of *Babesia* spp. and/or *Anaplasma* spp. in oversea imported reindeer specimens. Imported specimens (n=7) were hospitalized with visible tick infestation (*Ixodes ricinus*) and signs of cachexia, anemia, and hemoglobinuria. Using blood smears, PCR, and BLAST comparisons, it was confirmed that the animals were infected with a French strain of *Anaplasma phagocytophilum* and *Babesia divergens* which is considered to be absent in the USA. We conclude that oversea importation of reindeers must be followed with a routine check for geographically-specific strains of pathogens from the place of origin. This monitoring process must be dynamic and according to recent reports of tick-borne pathogens.

Key words: *Anaplasma, Babesia, Ixodes ricinus*, reindeer

INTRODUCTION

Babesia divergens, Babesia spp. EU1, *Borrelia* (*B. burgdorferi* s.l.), *Anaplasma* (*A. phagocytophilum*) and *Mycoplasma wenyonii* (or other haemoplasma closely related to) are the main pathogens transmitted by *Ixodes ricinus* to ruminants in Europe (1, 2, 3, 4). These *Ixodes*-related pathogens are geographically related to forest pastures are therefore most highly present in wild-animal ruminant species (5, 6). Transport of such animals may contribute to wide dispersion of local geographical strains of these pathogens. Babesiosis in reindeer has been related either to *Babesia odocoilei* (USA) or *B. divergens* (Europe) (7), or more recently to *Babesia* spp. EU1 (also known as *Babesia venatorum*) in the Netherlands (8). Moreover, up to 5 different *Babesia* species (*B. venatorum*, *B. capreoli*, *B. capreoli*-like, *B. odocoilei*-like and *B. divergens*) have been identified in asymptomatic captive reindeer in Germany (9). Infection by *Anaplasma* spp. is less reported despite the fact that *A. phagocytophilum* and *A. ovis* can infect reindeer (10, 11, 12).

We hypothesized that French strains of *Babesia* spp. or *Anaplasma* spp. may be diagnosed in imported, tick infested or non-infested reindeer specimens from an oversea geographical origin. Therefore, the aim of the current study was to investigate the correlation between severe clinical manifestation in imported reindeer specimens from the USA infested by *Ixodes* spp. and concomitant infection with *Babesia* spp. or *Anaplasma* spp.

MATERIAL AND METHODS

The study was conveyed on seven adult captive reindeers (*Rangifer tarandus tarandus*) that were hospitalized with medical history of anorexia, depression, pyrexia, and significant loss of weight.

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They have been imported from the USA approximately 4 weeks prior the hospitalization. Three were admitted dead, two died within a few hours after admission, and two were successfully treated. The deceased animals were processed on necropsy examination.

Blood smears (Microscope Nikon type 104, 40x) and coprology were performed on the treated animals. Blood cell count, hematocrit and hemoglobin were also performed (Hematology analyser XT 200 iV, Sysmex France, F-95944 Roissy). Multiple PCRs on blood samples was performed for *Anaplasma* (1, 12, 14, 15) and *Babesia* (16), followed by sequence analysis and BLAST comparison with sequence databanks.

DNAs were extracted from 200 µl of EDTA blood samples collected from reindeer using a QIAamp DNA Blood Mini Kit (QIAGEN® France-91974 Courtabeuf), eluted with 100 µl of buffer AE and stored at – 20 °C until use.

For *Anaplasma* spp., 5 distinct PCR assays were employed through two steps (as described in 12, 14, and 15). First, the DNA extracts were screened with broad-spectrum PCR primers targeting 16S rRNA gene of *Anaplasmataceae*, and then with primers targeting major surface protein 4 gene: *msp4*. Secondly, positive samples were subjected to confirmation by 3 amplification assays: PCRs targeting *groESL* heat shock operon, *ankA* gene, and citrate synthase gene: *gltA*. For *Babesia* a genus-specific PCR based on the amplification of a fragment of an 18S rRNA gene was performed, using BJ1 and BN2 primers (as described in 16).

PCR assays were performed on an Eppendorf® Mastercycler ep-Gradient thermocycler (Eppendorf France-78360 Montesson).

PCR products were analyzed by gel electrophoresis in 2% agarose (SYBR® Safe DNA gel stain, Invitrogen, Carlsbad, USA).

Sequence analysis was performed with NCBI blast tools (see: https://blast.ncbi.nlm.nih.gov/).

Table 1. Blood analysis from reindeer USA Texas 109 (leukogram, red cell count, hemoglobin, clinical chemistry)

Parameter	Value
Erythrocytes (10^{12}/L)	5.80 (10.70)
Haematocrit (%)	33 (47)
Haemoglobin (g/dL)	10.20 (17.30)
White blood cells (10^9/L)	5.53 (2.96)
Neutrophils (10^9/L)	4.90 (1.91)
Lymphocytes (10^9/L)	0.48 (0.90)
Monocytes (10^9/L)	0.05 (0.01)
Eosinophils (10^9/L)	<0.05 (0.00)
Basophils (10^9/L)	<0.05 (0.05)
Platelet count (10^9/L)	51.00 (235.00)
Fibrinogen (g/L)	4.61 (NA)
Glucose (mmol/L)	3.97 (6.70)
Urea (mmol/L)	13.60 (4.50)
Sodium (mmol/L)	144.00 (144.00)
Potassium (mmol/L)	3.70 (3.60)
Chloride (mmol/L)	103.00 (101.00)
Bicarbonate (mEq/L)	27.00 (NA)
Protein: total serum (g/L)	64.70 (62)
Albumin (g/L)	22.60 (NA)
Aspartate aminotransferase (IU/L)	389 (104)
Creatine phosphokinase (IU/L)	327 (356)
Gamma glutamyl transferase (IU/L)	168 (24)

In brackets: mean values adapted from Miller et al (13). NA: Not available. Hematology analyser XT 200 iV, Sysmex France, F-95944 Roissy. Biochemistry analyser VetTest, Iddex Europe, Hoofddorp, 2132 LR, The Netherlands

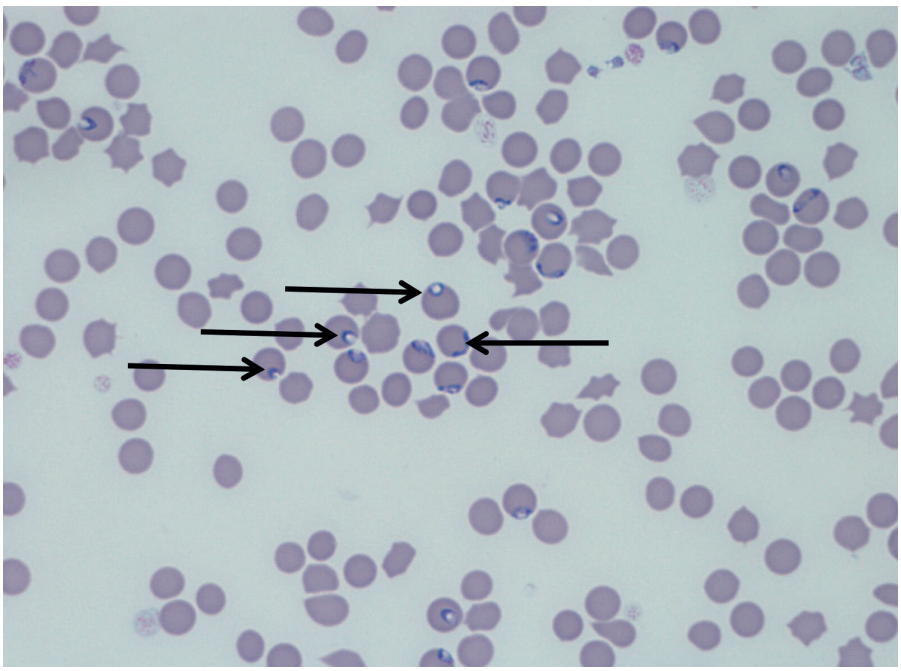

Figure 1. Multiple protozoa (arrows: pear-shaped intra-erythrocytic parasites) in a blood smear (from reindeer USA Texas 109) identified as *Babesia divergens*. Microscope Nikon type 104, 40x

RESULTS

Clinical examinations revealed cachexia, dehydration (estimated >10%), polypnea (>50 movements per minute), tachycardia (>90 beatings per minute), and hemoglobinuria. The color of mucosae was normal to pale, without signs of icterus. Tens to thousands of engorged *Ixodes ricinus* were found on each animal (from reindeer 172: 34 ticks, to reindeer 282: countless >1000 ticks).

Blood analysis revealed moderate anemia (Table 1).

Blood smears revealed intra-erythrocytic protozoa morphologically identified as *Babesia divergens* (Fig. 1).

Another blood smear a few hours after revealed the presence of intragranulocytic morulae identified as *Anaplasma phagocytophilum* (Fig. 2).

PCR results confirmed the morphological, bacteriological (Table 2) and parasitological findings (data not shown). After sequencing 5 genes of *A. phagocytophilum* (Genbank accession numbers JX841250 to JX841254, respectively), BLAST analyses of the sequences confirmed that the isolate was 100% identical to a French *A. phagocytophilum* isolate (strain BOV 10_179 CCXQ01000001 as deposited in the European Nucleotide Archive (see: https://www.ebi.ac.uk/ena/data/view/CCXQ01).

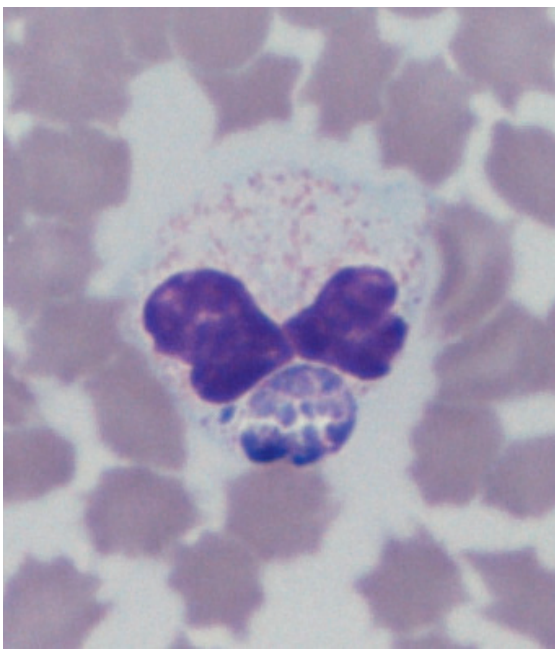

Figure 2. *Anaplasma phagocytophilum* morula (arrow: blackberry-shaped intracytoplasmic foreign body) in a neutrophilic granulocyte (reindeer USA Texas 109). Microscope Nikon type 104, 100x

Table 2. Amplification of 4 genes and partial 16S rRNA from *Anaplasma phagocytophilum* isolated in reindeer USA Texas 109: primers used and fragment length

Gene or genome portion	Primers (name and sequence)	Fragment length (base pair)
16S rRNA	EHR16SD : GGT ACC YAC AGA AGA AGT CC EHR16SR : TAG CAC TCA TCG TTT ACA GC	346 bp
msp4	MAP4AP5 : ATG AAT TAC AGA GAA TTG CTT GTA GG MSP4AP3 R : TTA ATT GAA AGC AAA TCT TGC TCC TAT G	849 bp
gltA	PCR1 : CS7F2 : ATG R*TA GAA AAW *GCT GTT TT HG1085R : ACT ATA CCK GAG TAA AAG TC PCR2 : F1b : GAT CAT GAR* CAR* AAT GCT TC AnaCS1076R : GAG TAA AAG TCG ACR*TTK*GG	450 bp
groESL	EEgro1F : GAG TTC GAC GGT AAG AAG TTC A AnaGroe240F: ATT AGY* AAG CCT TAT GGGTC	500 bp
ankA	U8: TAA GAT AGG TTT AGT AAG ACG 1Rmod: CTT AGT GCT TCA GCG GTC AG	350 bp

DISCUSSION

We found that all animals suffered from coinfection with the two major pathogens transmitted in Western Europe to ruminants by *Ixodes ricinus* (17). Poor nutritional condition, parasitism and transport-related stress (such as "shipping fever" documented in cattle) were the factors contributing to the severity of the disease.

Babesia spp. infection in reindeer is not considered to have frequent clinical manifestation. In the USA, *B. odocoieli* is correlated with a high mortality rate in reindeers (18), but it can affect other wild ruminants as well (elk - *Cervus elaphus elaphus*, and white-tailed deer - *Odocoielus virginianus*) (19, 20). A larger *Babesia* has also been described in one fatal case in a reindeer (20). *B. divergens* infects ruminants in Europe but has never been reported in the USA. *B. divergens*-like/MO-1 in the USA is a distinct strain of *B. divergens*, which has a lower infection rate in cattle, and distinct morphology when grown *in vitro* (21, 22, 23).

In early reports in Europe, infection of roe deer with *Babesia* spp. was supposed to result in subclinical to mild symptoms (11, 24). It is possibly due to the lack of exposure to tick bites under natural conditions, as reindeers seem to be extremely sensitive to most tick-borne pathogens (7, 19). In Europe, babesiosis in reindeer is induced by the very common *B. divergens* (7), but some protozoa close to *B. odocoieli* have also been detected in European ticks collected on wild cervids (5, 24). A larger *Babesia* (*B. jakimovi*) has also been described in naturally infected reindeers in Siberia. The zoonotic large *Babesia* spp. EU1 has been reported in *Ixodes ricinus* in a roe deer (3, 25, 26, 27), and in a reindeer (8). *B. divergens*, *Babesia* sp. EU1 and *B. odocoieli* are genetically and/or morphologically similar (3, 26, 27, 28). Many of "*Babesia divergens*" infections in European cervids may have been also caused by *B. capreoli* (3, 29). Hence, formal molecular identification of *Babesia* sp. in wild ruminants is routinely performed (12).

Anaplasma ovis infection may result in a severe disease in reindeer (10). The vectors associated with this bacterium are not *Ixodes* spp (30). In our case, no specific sequence of the bacterium was found (30).

Disease due to *Anaplasma phagocytophilum* infection has not been fully described in reindeer, even if the infection seems moderately prevalent (12). The infection in domestic and wild ruminants results in a seldom fatal disease unless complicated by other infections (2, 31). In our clinical observations, we hypothesized that the severe clinical manifestations in reindeer are a result of coinfection with *Babesia divergens*. Contributing factors were the supposed immune naivety and the shipping stressors.

Anaplasma phagocytophilum and/or *Babesia divergens* are frequently related to *Ixodes ricinus* infestations in Europe. The findings for *Anaplasma* range from 2 to 45% in various European countries (12, 32), for *Babesia divergens* from 0.9 to 6.7% (33), for both pathogens from to 4 to 8% in Germany (12, 34), and up to 46.7% in Poland (6). These rates are correlated with the tick life-stage and sex, detection method, and geography (country) (4). The tick population in one geographical zone may

contain one or more infective agents (6, 17, 35). The transmission cycle of *Anaplasma phagocytophilum* is not fully elucidated in Europe. If the documented vector is *Ixodes ricinus*, the reservoir is not precisely known (31, 34, 36, 37).

Tick bites in susceptible or weakened and immunologically compromised animals may therefore result in one or multiple infections. When introducing wild or domestic ruminants in a new pasture, a farm or country, it is important to consider which disease and which parasite they may introduce, but it is also imperative to consider the danger they incur when facing new parasites and new microbial pathogens.

CONCLUSION

This study identified the presence of *Babesia divergens* absent in the USA and French strain of *Anaplasma phagocytophilum* in reindeer specimen infested with *Ixodes ricinus* and imported from the USA. This concludes that tick-borne pathogens can be widely dispersed via vector transmission. The importation and transportation of wild animals is a significant risk factor.

Blood smears and molecular identification methods are effective in detection of tick-borne pathogens in suspect wild ruminants and should be routinely performed and updated.

CONFLICT OF INTEREST

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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AUTHORS' CONTRIBUTIONS

LR performed the clinical examination, necropsies, blood smears, blood analysis and partially the PCR analysis. RPM performed the morphological diagnosis from the blood smears and part of PCR and blast analysis. Both authors contributed equally in writing this manuscript.

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