

Research Article

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## The first detection of species of *Babesia* Starcovici, 1893 in moose, *Alces alces* (Linnaeus), in Norway

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**Abstract:** Babesiosis is an emerging zoonotic disease and various wildlife species are reservoir hosts for zoonotic species of *Babesia* Starcovici, 1893. The objective of the present study was to investigate the presence and prevalence of *Babesia* spp. in moose *Alces alces* (Linnaeus) in two regions of Norway. A total of 99 spleen samples were collected from animals of various ages from an area with the occurrence of the tick *Ixodes ricinus* (Linnaeus, 1758), and from an area where the ticks are known to be absent. Infection was detected by the amplification of different regions of the 18S rRNA gene by using two different PCR primer sets specific of *Babesia*. *Babesia* spp. were found in the spleen samples of four moose. All *Babesia*-infected animals were from an area where ticks occur, with an infection rate of 6% (4 of 70). *Babesia*-positive samples were obtained from a five-month old moose calf and three adults. Two *Babesia* species, *Babesia capreoli* (Enigk et Friedhoff, 1962) and a *B. odocoilei*-like, were identified. Co-infection with *Anaplasma phagocytophilum* was obtained in two animals. This is the first report of the occurrence of *B. capreoli* and *B. odocoilei*-like species in moose.

**Keywords:** *Babesia capreoli*, *Babesia odocoilei*-like, 18S rRNA gene, *Ixodes ricinus*, zoonosis

Ticks and tick-borne diseases in Europe have recently shown expansion in their distribution (Gray et al. 2009). Climatic and environmental changes are affecting the habitats and the structure of wildlife populations and are important drivers of tick population dynamics and geographic distribution. The tick *Ixodes ricinus* (Linnaeus, 1758) has been shown to be involved in the transmission of a range of zoonotic pathogens such as tick-borne encephalitis virus, bacteria causing Lyme disease and granulocytic anaplasmosis in humans and animals, as well as blood parasites such as intraerythrocytic species of *Babesia* Starcovici, 1893. Babesiosis is a worldwide tick-borne hemoprotozosis caused by intraerythrocytic protozoan parasites of the genus *Babesia*. The parasites infect red blood cells of vertebrate hosts and are transmitted by ixodid ticks. In Europe, babesiosis has been reported both in humans and some domestic and free-living mammals (Herwaldt et al. 2003). In Norway, one case of severe human babesiosis due to *Babesia divergens* (M'Fadyean et Stockman, 1911) has been documented, and seroprevalence of this species in cattle is known to be high (Hasle et al. 2010, Mørch et al. 2015).

To date, four species of *Babesia* have been described from wild European cervids: *B. venatorum* Herwaldt, Cacciò, Gherlinzoni, Aspöck, Slemenda, Piccaluga, Martinelli, Edelhofer, Hollenstein, Poletti, Pampiglione, Löschenberger, Tura et Pieniazek, 2003 (first known as *Babesia*

sp. EU1; Herwaldt et al. 2003), *B. divergens*, *B. capreoli* (Enigk et Friedhoff, 1962) and *B. odocoilei*-like (Duh et al. 2005, Bonnet et al. 2007a, Malandrin et al. 2010, Silaghi et al. 2011, Zintl et al. 2011, Zanet et al. 2014). *Babesia venatorum* has been identified in roe deer, *Capreolus capreolus* (Linnaeus) (Duh et al. 2005, Bonnet et al. 2007a). *Babesia divergens* and *B. capreoli* have been reported from roe deer, red deer, *Cervus elaphus* Linnaeus, reindeer, *Rangifer tarandus* (Linnaeus), alpine chamois, *Rupicapra rupicapra* (Linnaeus), and alpine ibex, *Capra ibex* (Linnaeus) (Langton et al. 2003, Malandrin et al. 2010, Zintl et al. 2011, Michel et al. 2014). However, the possible role of moose, *Alces alces* (Linnaeus), as a reservoir for *Babesia* spp. remains unknown. The aim of the present study was thus to investigate the occurrence and the diversity of *Babesia* spp. in moose to assess the role of this cervid as a possible reservoir for zoonotic babesiosis.

### MATERIALS AND METHODS

Spleen samples from 99 moose of different ages and sex were collected during the hunting seasons in 2013 and 2014 in Aust-Agder County (n = 70), an area where *Ixodes ricinus* ticks are present, and Oppland County (n = 29), an area where ticks are known to be absent. All spleen samples were kept frozen at -20 °C until analysed.

DNA from the spleens was extracted with the Genomic DNA Purification Kit K0512 (Thermo Fisher Scientific Baltics, Vilni-

**Table 1.** PCR conditions and primers used for the detection of *Babesia* spp.

Primer		Gene (bp)	Ds	As	Es	Cycles	Reference
1 <sup>st</sup> protocol							
BS1	External	18S rRNA (1 304 bp)	94 °C 1 min	58 °C 1 min	72 °C 1 min 30 s	35	Rar et al. 2005, 2011
5'-GACGGTAGGGTATTGGCCT							
BS2							
5'-ATTCACCGGATCCACTCGATC							
PiroA							
5'-ATTACCCAATCCTGACACAGGG	Internal	18S rRNA (380 bp)	94 °C 1 min	64 °C 1 min	72 °C 1 min 30 s	35	Armstrong et al. 1998, Rar et al. 2011
PiroC							
5'-CCAACAAAATAGAACCAAAGTCCTAC							
2 <sup>nd</sup> protocol							
Babfor	External	18S rRNA (652 bp)	95 °C 1 min	53 °C 1 min 30 s	72 °C 1 min 30 s	35	Zintl et al. 2011
5'-GACTAGGGATTGGAGGTC							
Babrev							
5'-GAATAATTCACCGGATCACTC							
BT2-F							
5'-GGAGTATGGTCGCAAGTCTG	Internal	18S rRNA (576 bp)	95 °C 1 min	53 °C 1 min 30 s	72 °C 1 min 30 s	35	Zintl et al. 2011
Babrev							
5'-GAATAATTCACCGGATCACTC							

Ds – denaturation step; As – annealing step; Es – elongation step.

us, Lithuania) according to the manufacturer’s instructions. To examine the presence of *Babesia*-DNA, two nested PCR protocols targeting different regions of the 18S rRNA gene were used (Table 1). Amplification was performed using the Mastercycler thermocycler (Eppendorf, Hamburg, Germany). Positive and negative controls were included in each PCR run. Amplification products were separated by electrophoresis on 1.5% agarose gel, stained with GelRed™ DNA-staining reagent (Biotium, Hayward, USA) and visualised under ultraviolet light.

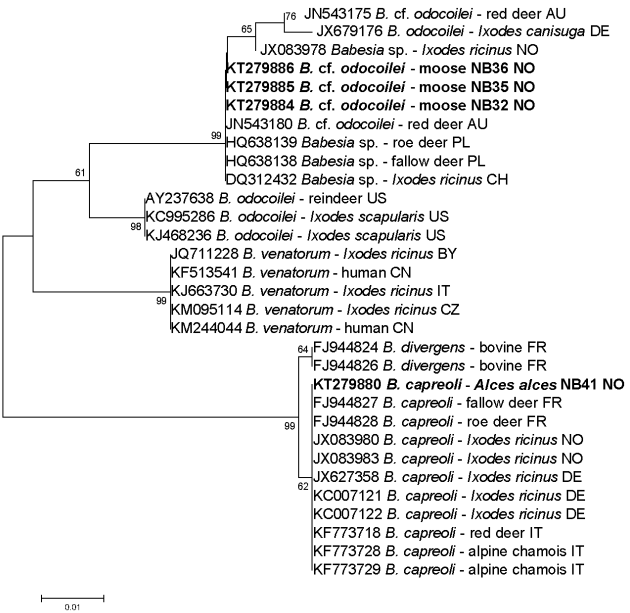
PCR-positive samples were confirmed by DNA sequencing of the partially amplified 18S rRNA gene of *Babesia* spp. Amplicons were cut out from agarose gel and purified using the GeneJET Gel Extraction Kit K0691 (Thermo Fisher Scientific Baltics). Forward primers for amplification were used as sequencing primers. Chromatograms were manually edited and trimmed prior to assembly. Nucleotide sequences were identified by the Basic Local Alignment Search Tool at the National Centre for Biotechnology Information using MEGA 6 software (Tamura et al. 2013).

A phylogenetic tree was constructed from the sequences of the 18S rRNA gene of *Babesia* spp. identified in the present study and those available from GenBank using the neighbour-joining method (Saitou and Nei 1987) with a bootstrap analysis of 1 000 replicates in MEGA 6. The partial nucleotide sequences of the *Babesia* spp. 18S rRNA gene were added to the GenBank database under the accession numbers: KT279879–KT279886.

**RESULTS**

*Babesia* spp. were found in four samples of moose spleens. All *Babesia*-infected animals were from the area where the tick *Ixodes ricinus* is present (Aust-Agder County; i.e. 4 of 70, prevalence 6%). *Babesia*-positive samples were obtained from a five-month old moose calf and three adults. The prevalence of *Babesia* spp. in Oppland County was 0% (n = 26).

Species of *Babesia* were identified by sequence-analysis of fragments amplified using both PCR protocols (Table 1). According to the 1<sup>st</sup> PCR protocol, 18S rDNA sequences (5' end of 18S rDNA region) derived from three *Babe-*



**Fig. 1.** Phylogenetic relationship of species of *Babesia* (samples from the present study bolded). Abbreviations: AU – Austria, BY – Belarus, CH – Switzerland, CN – China, CZ – Czech Republic, DE – Germany, FR – France, IT – Italy, NO – Norway, PL – Poland, US – United States of America.

sia-positive samples (isolates NB32, NB35 and NB36) were designated as *B. odocoilei*-like (Fig. 1). The sequences were identical to each other and showed 100% similarity to a sequence of *Babesia* cf. *odocoilei* (Emerson et Wright, 1970) (GenBank Accession No. JN543180) and to an unidentified species of *Babesia* (*Babesia* sp., GenBank Accession No. HQ638138) from red deer in Austria and Ireland, respectively (Fig. 1). These sequences were also identical to *Babesia* sp. from ticks feeding on red deer in Switzerland, which was previously described as a novel *Babesia* sp. genotype CH1 (GenBank Accession No. DQ312432) closely related to *B. odocoilei* reported from North Amer-

**Table 2.** Identification of species of *Babesia* species in moose from Norway.

Sample ID	GenBank ID 5'-end	GenBank ID 3'-end	Identified according to 1 <sup>st</sup> protocol	Identified according to 2 <sup>nd</sup> protocol
NB32	KT279884	KT279881	100% identical with <i>B. cf. odocoilei</i>	97% identical with <i>B. odocoilei</i> , <i>B. venatorum</i>
NB35	KT279885	KT279882	100% identical with <i>B. cf. odocoilei</i>	97% identical with <i>B. odocoilei</i> , <i>B. venatorum</i>
NB36	KT279886	KT279883	100% identical with <i>B. cf. odocoilei</i>	97% identical with <i>B. odocoilei</i> , <i>B. venatorum</i>
NB41	KT279880	KT279879	100% identical to <i>B. capreoli</i>	100% identical with <i>B. capreoli</i> , <i>B. divergens</i>

ica. Sequence analysis of the remaining sequence (isolate NB41) revealed 100% identity to *B. capreoli* (GenBank Accession Nos. KF773728, KF773729, JX083980).

According to the 2<sup>nd</sup> PCR protocol, BLAST search on 3' end of 18S rDNA region revealed a 100% similarity of isolates NB32, NB35 and NB36 with *Babesia* sp. derived from the tick *I. ricinus* in Norway (GenBank Accession No. JX042313) and *Babesia* sp. from red deer in Ireland (GenBank Accession No. GU475475). These sequences showed a 97% similarity with *B. odocoilei* described from elk (*C. elaphus canadensis* Erxleben) in Canada. Analysis of the sequence of NB41 sample of this region revealed a 100% similarity with *B. divergens* (GenBank Accession No. JX042324, JX042329 and GQ304524-5) and *B. capreoli* (GenBank Accession No. GQ304526 and FJ944827-8) as this region of 18S rDNA is identical for both species.

## DISCUSSION

Species of *Babesia* are among the most common hemoparasites in the world (Yabsley and Shock 2013). These parasites have a wide host range, including hundreds of species of mammals. Humans can serve as accidental hosts for numerous species of *Babesia* (see Yabsley and Shock 2013). Transovarial transmission of some *Babesia* spp. has been documented in *Ixodes ricinus* (see Bonnet et al. 2007b, 2009).

*Ixodes ricinus* is ubiquitous in southern Norway with the highest density near the coast (Mehl 1983, Jore et al. 2011). During the past decades, tick abundance has increased and its distribution area expanded further north and to higher altitudes (Jore et al. 2011, Medlock et al. 2013, Jore et al. 2014). The overall prevalence of *Babesia* spp. in questing *I. ricinus* ticks in Norway was 0.9% and several species such as *B. venatorum*, *B. divergens*, *B. capreoli* and *Babesia* sp. were identified (Radzijeuskaja et al. 2008, Øines et al. 2012).

We detected a relatively low prevalence of DNA of *Babesia* spp. in moose spleen if compared with what was previously observed in red deer (from 11% in Switzerland to 47% in Ireland) and roe deer (from 9% in Spain to 7% in Slovenia) (Silaghi et al. 2011).

Although initially specificity of *Babesia* spp. for the vertebrate host was described as restricted, the development of molecular tools allowed detecting some species of *Babesia* in a wider range of vertebrate hosts than was thought previously (Chauvin et al. 2009). *Babesia divergens* was originally described as a parasite of cattle in temperate regions, but later was identified in splenectomised humans, ungulates such as roe deer, red deer, fallow deer, moufflon and non-splenectomised rein deer and sheep (Chauvin et al. 2009). The ability of merozoites of *Babesia*

spp. to invade erythrocytes from a wide range of animal species was demonstrated in experimental studies and suggested possibilities for these parasites to adapt and develop in new hosts (Chauvin et al. 2009). *Ixodes ricinus* ticks parasitise on a wide range of vertebrate hosts and could harbour/carry *B. divergens*, *B. venatorum*, *B. capreoli* and *B. odocoilei*-like species (Øines et al. 2012), thus providing possibilities for efficient transmission of these *Babesia* spp. to new potential hosts such as moose.

The evolution of new strains or species of *Babesia* depends on the capacity of particular species of *Babesia* to develop in a new host. *Babesia* spp. have developed strategies to avoid the immune response, which enables them to survive inside the vertebrate host. The humoral immune response studied for some of *Babesia* spp., *B. ovis* (Babes, 1892), *B. divergens*, *B. canis* (Piana et Galli-Valerio, 1895) or *B. gibsoni* (Patton, 1910), showed the production of antibodies beginning approximately 7 days after infection and persisting for several months (Chauvin et al. 2009).

In the present study, we used two different PCR protocols. The alignment of 410 bp sequences in the 3' end of 18S rDNA of *Babesia* (2<sup>nd</sup> protocol, Table 1) showed that the sequence of NB41 individual is identical to those of *B. divergens* and *B. capreoli*. The differentiation between *B. capreoli* and *B. divergens* is difficult due to their morphological similarities and it is also complicated due to the high percentage of identity between the sequences of their respective 18S rRNA genes (Lempereur et al. 2012). In this study, identification of *B. capreoli* was based on the difference of two nucleotide bases on the amplified fragment of the 18S rRNA gene at positions 631 and 663 (Malandrin et al. 2010; Table 2).

*Babesia capreoli* was identified in the spleen of one moose (NB41, Fig. 1). This species is known to circulate among wild cervids with silent babesiosis (Penzhorn 2006). Babesiosis caused by *B. capreoli* in humans or livestock remains unknown (Malandrin et al. 2010). To the best of our knowledge, *B. capreoli* infection has not been previously reported in moose. We detected *B. odocoilei*-like species in three moose, which is the first detection of this species in moose. *Babesia odocoilei* is a parasite of North-American white-tailed deer, *Odocoileus virginianus* (Zimmermann), elk and caribou, *R. tarandus caribou* (Linnaeus), transmitted by ticks, *Ixodes scapularis* Say, 1821 (Holman et al. 2000). In Europe, *Babesia* sp. closely related to this parasite has been detected in *I. ricinus* and red deer was suggested as a possible reservoir (Duh et al. 2001, Herwaldt et al. 2003, Hilpertshauser et al. 2006, Zintl et al. 2011, Øines et al. 2012).

The pathogenicity of *Babesia* spp. differs between ungulate species. The occurrence of silent *Babesia* spp. in



fection in free-ranging cervids has been demonstrated in numerous studies, and it has been suggested that clinical babesiosis is rarely observed in wild ruminants (Penzhorn 2006). Fatal babesiosis due to *B. divergens*/*B. capreoli*-like infection has been described in chamois (Hoby et al. 2009).

Several recently published reports have indicated that *Babesia* infection due to *B. capreoli* and *B. venatorum* may have contributed to the mortality of roe deer, reindeer and alpine chamois (Kik et al. 2011, Michel et al. 2014). Severe infections and *Babesia*-related mortalities were found in reindeer due to *B. divergens* and *B. odocoilei* (see Langton et al. 2003, Barlett et al. 2009). However, it was noted that wild animals such as reindeer, when removed from their natural habitats (where babesiosis is not endemic)

and placed in captivity in non-native areas, are not adapted to *Babesia* parasites, and therefore may become severely infected and demonstrate clinical signs of babesiosis (Penzhorn 2006).

All 99 spleen samples of moose were also screened for *Anaplasma phagocytophilum* in another study (Pūraitė et al. 2015). Co-infection with *A. phagocytophilum* was recorded in two of the four moose, which were positive for *Babesia* spp. Both of the co-infected animals harboured *B. odocoilei*-like species. We thus conclude that moose may play a certain role in the circulation of *B. capreoli* and *B. odocoilei*-like species in Norway.

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